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(57) Abstract

This invention provides a novel protein designated FADD, FADD fragments, and anti-FADD antibodies which are useful to modulate FADD-associated cellular functions such as apoptosis. Also provided are nucleic acid molecules coding for these proteins and antibodies as well as process for making these compositions. Further provided are diagnostic and therapeutic utilities for these compositions.

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METHODS AND COMPOSITIONS FOR REGULATING FADD

This application is a continuation-in-part of applications U.S. Serial Nos. 08/416,379 and 08/443,982, filed April 3, 1995 and May 18, 1995, respectively, the contents of which are hereby incorporated by reference into the present disclosure.

This invention was made in part with support from the United States government under Grant No. CA 64803 from the National Institutes of Health. Accordingly, the United States government has certain rights in this invention.

Field of the Invention

This invention relates to FADD-mediated cellular functions and methods for the regulation of FADD-mediated cellular functions in a population of cells.

Background of the Invention

Programmed cell death (PCD) is a physiologic process essential to the normal development and homeostatic maintenance of multicellular organisms (reviewed in Vaux et al. (1994) Cell 76:777-779 and Ellis et al. (1991) Ann. Rev. Cell Biol. 7:663-698). Apoptosis, often equated with PCD, refers to the morphologic alterations exhibited by "actively" dying cells which include cell shrinkage, membrane blebbing and chromatin condensation. (For a general review of apoptosis, see Tomei, L.D. and Cope, F.O. Apoptosis: The Molecular Basis of Cell Death (1991) Cold Spring Harbor Press, N.Y.; Tomei, L.D.; Cope, F.O. Apoptosis II: The Molecular Basis of Apoptosis in Disease (1994) Cold Spring Harbor Press, N.Y.; Duvall and Wyllie (1986) Immun. Today 7(4):115-119 and Cohen (1993) Immunol. Today 14:126-130.) In contrast, necrosis, sometimes referred to as accidental cell death, is defined by the swelling and lysis of cells that are exposed to t xic stimuli.

Indeed, one of the major characteristics of AIDS is the gradual depletion of $CD4^+$ T lymphocytes during the development of the disease. It is therefore advantageous to have compositions and methods which will manipulate apoptotic cell death.

Several mechanisms, including apoptosis, have been suggested to be responsible for the CD4+ cell depletion. It is speculated that apoptotic mechanisms might be mediated either directly or by the virus replication as a consequence of the HIV envelope gene expression, or indirectly by priming uninfected cells to apoptosis when triggered by different agents.

The depletion of CD4+ T cells results in the impairment of the cellular immune response. It has been reported that an inappropriate activation-induced T cell PCD causes the functional and numerical abnormalities of T_H cells from HIV-infected patients, that leads to the near collapse of the patient's immune system. (Brunner, T. et al. (1995) Nature 373:441-444; Dhein, J. et al. (1995) Nature 373:438-441; and Ju, S-T. et al. (1995) Nature 373:444-448).

Therefore, it is advantageous to block apoptosis and the ensuing depletion of T cells, especially in HIV infected individuals. Accordingly, a need exists to maintain T cell function and viability in HIV infected individuals and to provide systems to screen for new drugs that may assist in maintaining the cellular immune response. This invention satisfies these needs and provides related advantages as well.

Summary of the Invention

This invention provides a novel purified protein, and recombinant proteins and polypeptides designated "FADD". Equivalents, as well as muteins, analogs and fragments thereof, of the proteins and polypeptides also are provided by this invention. "FADD" has been shown to have the ability to modulate cellular functions associated with the Fas/CD95/APO-1 and TNF Necrosis Receptor–1 (TNFR-1) cell surface receptors, and in particular, apoptosis induced by activation of the receptors by binding of their respective ligands. In some embodiments, the proteins and polypeptides augment apoptosis and in other embodiments, they inhibit or prevent apoptosis. The proteins and polypeptides are further characterized by having the ability to bind the cytoplasmic region of a Fas receptor.

Also provided by this invention are nucleic acid molecules coding for the above proteins and polypeptides. Further provided herein are antibodies capable of specifically forming an antibody complex with the FADD proteins and polypeptides described herein, as well as the hybridoma cell lines that produce the antibodies.

This invention further provides an agent characterized by having the ability to inhibit the binding of a FADD protein or polypeptide of this invention to the cytoplasmic domain of a Fas receptor and its mediation of TNFR-1 induced apoptosis.

Methods of modulating a cellular function regulated by the Fas and TNFR-1 receptor pathway in a suitable cell also are provided herein. The methods comprise introducing into the cell a FADD nucleic acid and growing the cell under suitable conditions such that the nucleic acid is transcribed and translated into FADD protein in the cell.

Additionally, methods for screening for an agent useful to modulate cellular function regulated by the FADD pathway, the method comprising the steps of: a) providing a cytoplasmic domain of the receptor bound to a solid support; b) contacting the agent to be tested with the receptor bound support of step a) under conditions favoring binding of the cytoplasmic domain to the receptor to FADD; c) contacting detectably-labeled FADD to the solid support of step b) under conditions favoring binding of the cytoplasmic domain of the receptor to FADD; d) detecting the presence of any complex formed between the receptor and FADD to form receptor-

FADD complex; e) the absence of complex being indicative that the agent inhibits binding of FADD to the receptor; and f) analyzing the results of step d) to determine how the agent modulates the cellular function regulated by the FADD pathway.

Further provided are methods for screening for an agent useful to modulate cellular functions regulated by the FADD pathway, the method comprising the steps of: a) providing a cytoplasmic domain of a FADD (e.g., Fas or TNFR-1) bound to a solid support; b) contacting detectably-labeled FADD to the solid support of step a) under conditions favoring binding of the cytoplasmic domain receptor to FADD; c) contacting the agent to be screened with the receptor bound support of step b) under conditions favoring binding of the cytoplasmic domain to the receptor to FADD; d) detecting the presence of any complex formed between the receptor and FADD to form a receptor-FADD complex; and e) the absence of complex being indicative that the agent competitively inhibits binding of FADD to the receptor; and f) analyzing the results of step e) to determine how the agent modulates the cellular function regulated by the FADD pathway.

Brief Description of the Figures

Figure 1 shows that full length FADD specifically interacts with the cytoplasmic domain of Fas in yeast. This figure shows the results of β-galactosidase filter assays performed on Y190 yeast expressing the GAL4 activation domain-FADD fusion protein and indicated heterologous GAL4 DNA binding domain fusion proteins.

Figures 2A and 2B show sequence analysis of FADD and its novel death domain. Figure 2A (Seq. ID Nos. 1 and 2) is the coding strand of the cDNA sequence of isolated FADD and the deduced amino acid sequence of the FADD protein product. The boxed nucleotides represent an in-frame stop codon 130 base pairs upstream of the initiator methionine. The 5' end of clones 8 and 15 isolated in the yeast two-hybrid screen are indicated with arrows. The death domain is underlined while the valine residue altered to an asparagine in FADDmt (Seq. ID Nos. 1 and 2) is indicated by the closed triangle. A potential poly (A) adenylation signal (ATTAAA) is overlined.

Figure 2B (Seq. ID Nos. 3 through 6) shows the death domain of FADD and its amino acid sequence homology to other death domains. Solid black shading refers to identical residues and gray shading indicates conservative amino acid substitutions relative to the sequence of FADD. The arrow indicates the amino acid residue, which when substituted by an asparagine, disrupts binding and/or signaling in the respective proteins.

Figures 3A and 3B show that FADD is expressed in a variety of tissues and developmental stages. In Figure 3A, a human adult tissue Northern blot (Clontech) was probed with FADD cDNA, PBL=peripheral blood leukocyte. Figure 3B is a human fetal Northern blot (Clontech) that was probed as in Figure 3A.

Figures 4A through 4C show the specific interaction of GST-Fas and GST-Fas-FD5 with *in vitro* translated FADD and FADD expressed in transfected 293T cells. Figure 4A is a schematic representation of the GST fusion proteins containing the cytoplasmic domains of Fas, Fas mutants, and TNFR-1. Amino acid residues are given for selected junctions and numbering is based on the mature form of the receptor. The *Lpr* mutant ($V^{238} \rightarrow N^{238}$) of Fas is represented by an asterisk. The gray shading represents the death domain of FAS. Binding of FADD to the various GST fusion proteins is depicted to the right and is based on data from B, below.

Figure 4B shows the interaction of *in vitro* translated, ^{35}S-labeled FADD with various GST fusion proteins immobilized on glutathione-Sepharose beads. After the beads were washed, retained FADD protein was analyzed by SDS-PAGE and autoradiography (upper panel). The gel was Coomassie stained and the bands representing the various GST fusion proteins were aligned to show equivalency of loading (lower panel).

In Figure 4C, 293T cells were transfected with HA-epitope tagged FADD (HA-FADD) and metabolically labeled with ^{35}S-methionine and cysteine. Detergent lysates were prepared and incubated with the various GST fusion proteins immobilized on glutathione-Sepharose beads. After washing, the complexed beads were dissociated and immunoprecipitated with an anti-HA (α-HA) antibody which should recognize HA-FADD. The samples were then analyzed by SDS-PAGE and

autoradiography (upper panel). The respective GST fusion proteins were shown as in B (lower panel).

Figures 5A through 5D show *in vivo* association of FADD with Fas and Fas-FD5. Figure 5A is a schematic representation of Fas and Fas mutants transfected into 293T cells. The black square represents the FLAG-epitope tag engineered 5 amino acids downstream of the putative signal sequence of Fas. The open rounded rectangles represent the 3 cysteine-rich subdomains of the extracellular domain of Fas, while the cytoplasmic residues contain the death domain (gray rectangle) and a putative negative regulatory domain (shaded oval). Residue numbering is based on the mature form of the receptor and the amino acid sequence is given for selected junctions. The Lpr mutant ($V^{238} \Rightarrow N^{238}$) of Fas is represented by an asterisk. *In vivo* FADD binding is described to the right of the schematic along with relative cell death caused by Fas and its mutants. Binding was determined by the method as described by Itoh et al. (1993) Cell 66:233-243.

For the results shown in Figure 5B, 293T cells were cotransfected with HA-FADD and FLAG-epitope tagged Fas and Fas mutants (as depicted in Figure 5A) and metabolically labeled with ^{35}S methionine and cysteine. Detergent lysates were then immunoprecipitated with anti-FLAG (α-FLAG) mAb and isotype-matched control antibody and analyzed by SDS-PAGE and autoradiography to show expression of FLAG-tagged Fas and Fas mutants. White asterisks indicate relative position of Fas and its mutants.

In the results shown in Figure 5C, 293T lysates (as in Figure 5B) also were immunoprecipitated with α-HA antibody to show HA-FADD expression.

Figure 5D shows the coimmunoprecipitation of FADD with Fas and mutants. A fraction of the α-HA immunoprecipitates (used in Figure 5C) were dissociated and reimmunoprecipitated with an α-FLAG antibody.

Figure 6 shows that FADDmt fails to bind Fas, suggesting a death domain to death domain interaction. 293T cells were transfected with AU1-epitope tagged FADD (AU1-FADD) or AU1-FADDmt metabolically labeled with ^{35}S-methionine and cysteine. Detergent lysates were prepared and incubated with various GST fusion proteins immobilized on glutathione-Sepharose beads. The samples were analyzed by SDS-PAGE and autoradiography (upper panel). The respective GST fusion proteins

are shown as in Figure 4B (middle panel). To show that equivalent amounts of AU1-FADD and AU1-FADDmt were expressed and subsequently incubated with the beads, an aliquot of the respective lysates was immunoprecipitated with α-AU1 antibody and visualized by SDS-PAGE and autoradiography (bottom panels).

Figures 7A through 7C show that expression of FADD in BJAB cells induces apoptosis which is inhibitable by *CrmA*. Shown in Figure 7A is a previously characterized BJAB cell line expressing *CrmA* (as described in Tewari et al. (1995) J. Biol. Chem. 270:3255-3260) and a corresponding vector control line which were transiently transfected with pCMV β-galactosidase in the presence or absence of an equimolar quantity of pcDNA3 AU1-FADD. The cells were cytocentrifuged, fixed, and stained for β-galactosidase (yellow) and with propidium iodide (red). Shown in Panel 1 is a vector control line transfected with β-galactosidase. Panel 2 shows vector control line transfected with β-galactosidase and pcDNA3-AU1-FADD. Panel 3 shows *CrmA*-expressing line transfected with β-galactosidase. Panel 4 shows the *CrmA*-expressing line transfected with β-galactosidase and pcDNA3-AU1-FADD.

In Figure 7B, at least 100 transfected cells, processed as in Figure 7A, were counted and designated as apoptotic or non-apoptotic as determined by cell morphology.

Figure 7C shows immunostaining of AU1-FADD (green) which was transiently transfected into a BJAB cell line expressing *CrmA*. Propidium iodide staining (red) reveals nuclei.

Figures 8A and 8B show biological and functional effects of expression of FADD and mutants in MCF7 cells. Figure 8A (left panel) shows overexpression of FADD and β-galactosidase in MCF7/mutant *CrmA* cells. In the right panel, overexpression of FADD and β-galactosidase in MCF7/*CrmA* cells is shown. Cells were stained with X-gal and examined by phase contrast microscopy. See Table 1 for details. Figure 8B is a schematic representation of FADD and mutants. Amino acid residues are given for selected junctions. The point mutation of FADD ($V^{121} \rightarrow N^{121}$) is represented by an asterisk. The gray and black rectangles represent the death domain of FADD and an AU1-epitope tag, respectively. Ability of the various mutants to induce cell death in MCF7 cells is described to the right of the schematic and is based on data from Table 1.

Figures 9A through 9D show that FADD mediates CD95 signal transduction. In Figure 9A, BJAB cells expressing FADD-DN are resistant to CD95-induced apoptosis. The indicated cell lines were incubated for hours with various concentrations of anti-Fas IgM and cell death assessed by nuclear morphology. At least 250 cells were counted in 3 independent experiments (mean ± SD). Expression of FADD-DN is shown in the photographic insets. FADD-DN migrates as a doublet around 18 kDa due to post -translational modification (see Kischel, F.C. (1995) EMBO 14:5579-5588). The TUNEL assay is shown in the graphical inset and at least 250 cells were counted in 3 independent experiments (mean ± SD). In Figure 9B, anti-CD95-induced ceramide generation is abrogated by FADD-DN. The indicated BJAB cell lines were treated with anti-Fas IgM (1μg/ml) for the various times listed and ceramide levels subsequently assessed (mean ± SD; n=3). Significant levels of ceramide could not be detected at 5-, 10-, 30- and 60- minute time points (inset). In Figure 9C, C_2-ceramide (C2), but not C_2- dihydroceramide (DHC2), can bypass the dominant negative effect of the FADD derivative. The cells characterized include BJAB-vector, BJAB-FADD-DN, and BJAB-sFADD-DN. As a control, cells were also exposed to the structurally-related inactive analog, C^2-dihydroceramide as described in Bielawska, A. et al. (1994) J. Biol. Chem. 268:26226–26232. Viabilities were not decreased significantly, thus validating the specificity of the cytotoxic effect of C_2-ceramide. The x axis refers to the concentration of synthetic ceramide used and the y axis refers to viability as assessed by MTT conversion. Viability is expressed as percentage of vehicle-treated control ± SEM. Results are representative of three independent experiments. In 9D, CD95-induced activation of the apoptotic protease Yama is blocked by FADD-DN. BJAB vector and BJAB-sFADD-DN were left untreated or treated with 100 ng/ml anti-FAS IgM for 18 hrs. Lysates were then run on a 15% gel and immunoblotted with polyclonal antibodies directed against the 17 kDa and 12 kDa subunits of Yama (upper panel). Cleavage of the death substrate poly (ADP-ribose) polymerase also was assessed (lower panel).

Figures 10A and 10B show that FADD mediates TNF-induced cell death, but not TNF-induced NF-κB activation. In Figure 10A, the stable cell lines utilized include MCF7-sFADD-DN which represents a pool of 9 resistant clones and a corresponding MCF7-vector control. Expression of FADD-DN is shown in the left

panels. FADD-DN migrates as a doublet around 18kDa possibly due to post-translational modification. The indicated cell lines were either 1 ft untreated (UnRx) or treated with anti-Fas IgM (250ng/ml) plus cycloheximide (CHX, 10μg/ml) or 100ng/ml TNF for 24 hours and stained with propidium iodide. Similar results were obtained using anti-APO-1 antibody plus soluble Protein A in the absence of CHX. Phase contrast micrographs are shown with corresponding confocal micrographs (insets) depicting nuclear morphology. In Figure 10B, MCF7-vector or MCF7-sFADD-DN cells were transfected with an NF-κB-dependent E-selectin-luciferase reporter construct (see Rothe et al. (1995) Science 269:1425–1427) and were either untreated or treated with TNF for 9 hrs. Luciferase activities were assessed as described in Rothe et al. (1995) *supra*, and values shown are mean ± SD of three independent experiments.

Figures 11A and 11B show a possible mechanism for the inhibitory action of FADD-DN. In Figure 11A, 293T cells were co-transfected with AU1-epitope tagged FADD constructs and FLAG-tagged constructs encoding CD95, TNFR-1 and B94. The cells were lysed and FADD or FADD-DN was immunoprecipitated with anti-FADD polyclonal antibody, run on a 15% SDS polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. Co-precipitating FLAG-Fas and FLAG-TNFR-1 were identified by immunoblotting with anti-FLAG antibody. B94, a 73 kDa protein known in the art and described in Sarma et al. (1992) J. Immunol. 269:3302-3312, did not co-precipitate with FADD, verifying the specificity of the protein-protein interaction. All transfected components were assessed for expression by immunoblotting cell lysates. In Figure 11B, a truncated derivative of FADD exerts a dominant negative effect by displacing endogenous FADD from activated CD95 and thereby inhibits DISC formation. BJAB-vector (upper panels) and BJAB-sFADD-DN cells (middle panels) were metabolically labeled (with ^{35}S cysteine and methionine), lysed with Triton X-100, immunoprecipitated with anti-APO-1/PA-Sepharose, and subsequently analyzed by 2D IEF/12% SDS-PAGE. As expected, the four CAPs (Cytotoxically-dependent APO-1-Associated Proteins, see Kirschel (1995) *supra*) as well as FADD-DN, failed to associate with the un-activitated (not oligomerized) APO-1. However, cells stimulated with anti-APO-1 for 5 min and then lysed, show association of the four CAP proteins with the activated receptor in vector transfected

cells. By contrast, in FADD-DN expressing cells, FADD-DN associated with the oligomerized APO-1 while the CAPs did not. The lower left panel is a schematic illustration of the migration positions of APO-1, CAPs, and FADD-DN. Large open arrow head: migration positions of endogenous FADD (CAP1, 2). Large closed arrow head: FADD-DN. Small open arrow head: CAP3 (26 kDa) and CAP4 (55 kDa). APO-1 runs as an array of spots around 54 kDa on the basic half of the gel. FADD-DN was identified in activated sFADD-DN cells by immunoblotting using anti-AU1 antibody (lower right panel).

Detailed Description of the Invention

Although the morphologic features of cell death are well described, the molecular mechanisms behind apoptosis remain undefined. Recent work on PCD in the nematode *Caenorhabditis elegans* suggests that CED-3 initiates the cell death program (Yuan et al. (1993) Cell 75:641-652). Sequence analysis revealed that CED-3 is similar to the mammalian interleukin-1β (IL-1β) converting enzyme (ICE); a cysteine proteinase involved in the processing and activation of pro-IL-1β to the active cytokine (Cerretti et al. (1992) Science 256:97-100 and Thornberry et al. (1992) Nature 356:768-774). Overexpression of ICE in mammalian cells induced apoptosis, suggesting that ICE, or a related protease, may be an essential component of the cell death pathway (Miura et al. (1993) Cell 75:653-660).

If a CED-3 like protease is presumed to be a distal effector of the mammalian cell death pathway, the proximal components that lead to its activation remained to be identified.

Two cell surface cytokine receptors, Fas/APO-1 antigen and the receptor for Tumor Necrosis Factor (TNF), have been shown to trigger apoptosis by natural ligands or specific agonist antibodies (Baglioni, C. (1992) The Molecules and Their Emerging Roles in Medicine (Raven Press, N.Y., N.Y.); Yonehara et al. (1989) J. Exp. Med. 169:1747-1756; Itoh et al. (1991) Cell 66:233-243; Trauth, B.C. et al. (1989) Science 245:301-305). The Fas antigen is involved in the negative selection of thymic T-lymphocytes and mice carrying a point mutation in the cytoplasmic domain of Fas exhibit a lupus-like lymphoproliferative autoimmune disorder (*Lpr*)

(Watanabe-Fukunaga et al. (1992) Nature 356:314-317). Recently, the Fas-mediated cell pathway has been implicated in the activation-induced death f T-cells (Dhein et al., J. (1995) *supra*; Brunner, T. et al. (1995); *supra*; and Ju et al. (1995) *supra*.) While the main activity of Fas is to trigger cell death, the TNF receptor (TNFR) can signal an array of diverse activities such as apoptosis, fibroblast proliferation, resistance to chlamidlae and synthesis of prostaglandin E_2 (Tartaglia, L.A. et al. (1992) Immunol. Today 13:151-153).

The activation of Fas and TNFR is caused by receptor aggregation mediated by their respective ligands or agonist antibodies. The signal is thought to be transduced by clustering of the intracellular domain (Boldin, M.P. et al. (1995) J. Biol. Chem. 270:387-391 and Song, H.Y. et al. (1994) J. Biol. Chem. 269:22492-22495) which encompasses a region which is significantly conserved in the Fas antigen as well as in TNFR-1 (Tartaglia et al. (1993) *supra* and Itoh, N. et al. (1993) J. Biol. Chem. 266:10932-10937). This shared "death domain" suggests that both receptors interact with a related set of signal transduction molecules that had, until this disclosure, remained unidentified.

Provided herein is the molecular cloning and characterization of "FADD" a Fas Associating protein with a novel Death Domain.

Definitions

The terms "proteins", "peptides" and "polypeptides" are used interchangeably and are intended to include molecules containing amino acids linearly coupled through peptide bonds. The amino acids can be in the L or D form so long as the biological activity of the polypeptide is maintained. For example, the protein can be altered so as to be secreted from the cell for recombinant production and purification. These also include proteins which are post-translationally modified by reactions that include glycosylation, acetylation and phosphorylation. Such polypeptides also include analogs, alleles and allelic variants which can contain amino acid derivatives or non-amino acid moieties that do not affect the biological or functional activity of the protein as compared to wild-type or naturally occurring protein. The term amino acid refers both to the naturally occurring amino acids and their derivatives, such as TyrMe and PheCl, as well as other moieties characterized by the presence of both an

available carboxyl group and an amine group. Non-amino acid moieties which can be contained in such polypeptides include, for example, amino acid mimicking structures. Mimicking structures are those structures which exhibit substantially the same spatial arrangement of functional groups as amino acids but do not necessarily have both the I-amino and I-carboxyl groups characteristic of amino acids.

"Muteins" are proteins or polypeptides which have minor changes in amino acid sequence caused, for example, site-specific mutagenesis or other manipulations; by errors in transcription or translation; or which are prepared synthetically by rational design. These minor alterations result in amino acid sequences wherein the biological activity of the protein or polypeptide is altered as compared to wild-type or naturally occurring polypeptide or protein. Examples of FADD muteins are FADDmt and AU1-N-FADD described below.

As used herein, the term "peptide bond" or "peptide linkage" refers to an amide linkage between a carboxyl group of one amino acid and the α-amino group of another amino acid.

As used herein, the term "hydrophobic" is intended to include those amino acids, amino acid derivatives, amino acid mimics and chemical moieties which are non-polar. Hydrophobic amino acids include Phe, Val, Trp, Ile and Leu. As used herein, the term "positively charged amino acid" refers to those amino acids, amino acid derivatives, amino acid mimics and chemical moieties which are positively charged. Positively charged amino acids include, for example, Lys, Arg and His.

"Purified" when referring to a protein or polypeptide, are distinct from native or naturally occurring proteins or polypeptides because they exist in a purified state. These "purified" proteins or polypeptides, or any of the intended variations as described herein, shall mean that the compound or molecule is substantially free of contaminants normally associated with the compound in its native or natural environment.

"Native" polypeptides, proteins, or nucleic acid molecules refer to those recovered from a source occurring in nature or "wild-type".

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

A "pharmaceutical composition" is intended to include the combination f an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, Remington's Pharm. Sci., 15th Ed. (Mack Publ. Co., Easton (1975)).

The term "nucleic acid" means single and double stranded DNA, cDNA, genome-derived DNA, and RNA, as well as the positive and negative strand of the nucleic acid that are complements of each other, including anti-sense RNA. A "nucleic acid molecule" is a term used interchangeably with "polynucleotide" and each refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides, or analogs thereof. It also includes known types of modifications, for example labels which are known in the art (e.g., Sambrook, et al. (1989) *infra.*), methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl carbamate, etc.), those containing pendant moieties, such as for example, proteins (including , e.g., nuclease, toxins, antibodies, signal peptides, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide. The polynucleotide can be chemically or biochemically modified or contain non-natural or derivatized nucleotide bases. The nucleotides may be complementary to the mRNA encoding the polypeptides. These complementary nucleotides include, but are not limited to, nucleotides capable of forming triple helices and antisense nucleotides. Recombinant polynucleotides comprising sequences otherwise not naturally occurring are also provided by this invention, as are alterations of wild type polypeptide sequences, including but not limited to, those due to deletion, insertion, substitution of one or more nucleotides or by fusion to other polynucleotide sequences.

A polynucleotide is said to "encode" a polypeptide if, in its native state or when manipulated by methods well-known to those skilled in the art, it can be transcribed and/or translated to produce a polypeptide or mature protein. Thus, the term polynucleotide shall include, in addition to coding sequences, processing sequences and other sequences that do not code for amino acids of the mature protein. The anti-sense strand of such a polynucleotide is also said to encode the sequence.

The term "recombinant" polynucleotide or DNA refers to a polynucleotide that is made by the combination of two otherwise separated segments of sequence accomplished by the artificial manipulation of isolated segments of DNA by genetic engineering techniques or by chemical synthesis. In so doing one may join together DNA segments of desired functions to generate a desired combination of functions.

An "analog" of DNA, RNA or a polynucleotide, refers to a macromolecule resembling naturally occurring polynucleotides in form and/or function (particularly in the ability to engage in sequence-specific hydrogen bonding to base pairs on a complementary polynucleotide sequence) but which differs from DNA or RNA in, for example, the possession of an unusual or non-natural base or an altered backbone. See for example, Uhlmann et al. (1990) Chemical Reviews 90:543-584.

"Isolated" when referring to a nucleic acid molecule, means separated from other cellular components normally associated with native or wild-type DNA or RNA intracellularly.

"Hybridization" refers to hybridization reactions can be performed under conditions of different "stringency". Conditions that increase the stringency of a hybridization reaction are widely known and published in the art: see, for example, Sambrook, et al., *infra*. Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25 °C, 37 °C, 50 °C, and 68 °C; buffer concentrations of 10 x SSC, 6 x SSC, 1 x SSC, 0.1 x SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalent using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours and washes of increasing duration, increasing frequency, or decreasing buffer concentrations.

"T_m" is the temperature in degrees Centigrade at which 50% of a polynucleotide duplex made of complementary strands hydrogen bonded in an antiparallel direction by

Watson-Crick base paring dissociates into single strands under the conditions of the experiment. T_m may be predicted according to standard formula; for example:

$$T_m = 81.5 + 16.6 \log [Na^+] + 0.41 (\%G/C) - 0.61 (\%F) - 600/L$$

where Na^+ is the cation concentration (usually sodium ion) in mol/L; (%G/C) is the number of G and C residues as a percentage of total residues in the duplex; (%F) is the percent formamide in solution (wt/vol); and L is the number of nucleotides in each strand of the duplex.

A "stable duplex" of polynucleotides, or a "stable complex" formed between any two or more components in a biochemical reaction, refers to a duplex or complex that is sufficiently long-lasting to persist between the formation of the duplex or complex, and its subsequent detection. The duplex or complex must be able to withstand whatever conditions exist or are introduced between the moment of formation and the moment of detection, these conditions being a function of the assay or reaction which is being performed. Intervening conditions which may optionally be present and which may dislodge a duplex or complex include washing, heating, adding additional solutes or solvents to the reaction mixture (such as denaturants), and competing with additional reacting species. Stable duplexes or complexes may be irreversible or reversible, but must meet the other requirements of this definition. Thus, a transient complex may form in a reaction mixture, but it does not constitute a stable complex if it dissociates spontaneously or as a result of a newly imposed condition or manipulation introduced before detection.

When stable duplexes form in an antiparallel configuration between two single-stranded polynucleotides, particularly under conditions of high stringency, the strands are essentially "complementary". A double-stranded polynucleotide can be "complementary" to another polynucleotide, if a stable duplex can form between one of the strands of the first polynucleotide and the second. A complementary sequence predicted from the sequence of a single stranded polynucleotide is the optimum sequence of standard nucleotides expected to form hydrogen bonding with the single-stranded polynucleotide according to generally accepted base-pairing rules.

A "sense" strand and an "antisense" strand when used in the same context refer to single-stranded polynucleotides which are complementary to each other. They may be opposing strands of a double-stranded polynucleotide, or one strand may be predicted

from the other according to generally accepted base-pairing rules. Unless otherwise specified or implied, the assignment of one or the other strand as "sense" or "antisense" is arbitrary.

A linear sequence of nucleotides is "identical" to another linear sequence, if the order of nucleotides in each sequence is the same, and occurs without substitution, deletion, or material substitution. It is understood that purine and pyrimidine nitrogenous bases with similar structures can be functionally equivalent in terms of Watson-Crick base-pairing; and the inter-substitution of like nitrogenous bases, particularly uracil and thymine, or the modification of nitrogenous bases, such as by methylation, does not constitute a material substitution. An RNA and a DNA polynucleotide have identical sequences when the sequence for the RNA reflects the order of nitrogenous bases in the polyribonucleotide, the sequence for the DNA reflects the order of nitrogenous bases in the polydeoxyribonucleotide, and the two sequences satisfy the other requirements of this definition. Where at least one of the sequences is a degenerate oligonucleotide comprising an ambiguous residue, the two sequences are identical if at least one of the alternative forms of the degenerate oligonucleotide is identical to the sequence with which it is being compared. For example, AYAAA is identical to ATAAA, if AYAAA is a mixture of ATAAA and ACAAA.

When comparison is made between polynucleotides, it is implicitly understood that complementary strands are easily generated, and the sense or antisense strand is selected or predicted that maximizes the degree of identity between the polynucleotides being compared. For example, where one or both of the polynucleotides being compared is double-stranded, the sequences are identical if one strand of the first polynucleotide is identical with one strand of the second polynucleotide. Similarly, when a polynucleotide probe is described as identical to its target, it is understood that it is the complementary strand of the target that participates in the hybridization reaction between the probe and the target.

A linear sequence of nucleotides is "essentially identical" or the "equivalent" to another linear sequence, if both sequences are capable of hybridizing to form duplexes with the same complementary polynucleotide. It should be understood, although not always explicitly stated that when Applicants refer to a specific nulceic acid molecule, its equivalents are also intended. Sequences that hybridize under conditi ns of greater

stringency are more preferred. It is understood that hybridization reactions can accommodate insertions, deletions, and substitutions in the nucleotide sequence. Thus, linear sequences of nucleotides can be essentially identical even if some of the nucleotide residues do not precisely correspond or align. Sequences that correspond or align more closely to the invention disclosed herein are comparably more preferred. Generally, a polynucleotide region of about 25 residues is essentially identical to another region, if the sequences are at least about 80% identical; more preferably, they are at least about 90% identical; more preferably, they are at least about 95% identical; still more preferably, the sequences are 100% identical. A polynucleotide region of 40 residues or more will be essentially identical to another region, after alignment of homologous portions if the sequences are at least about 75% identical; more preferably, they are at least about 80% identical; more preferably, they are at least about 85% identical; even more preferably, they are at least about 90% identical; still more preferably, the sequences are 100% identical.

In determining whether polynucleotide sequences are essentially identical, a sequence that preserves the functionality of the polynucleotide with which it is being compared is particularly preferred. Functionality can be determined by different parameters. For example, if the polynucleotide is to be used in reactions that involve hybridizing with another polynucleotide, then preferred sequences are those which hybridize to the same target under similar conditions. In general, the T_m of a DNA duplex decreases by about 10 °C for every 1% decrease in sequence identity for duplexes of 200 or more residues; or by about 50 °C for duplexes of less than 40 residues, depending on the position of the mismatched residues (see, e.g., Meinkoth et al.). Essentially identical or equivalent sequences of about 100 residues will generally form a stable duplex with each other's respective complementary sequence at about 20 °C less than T_m; preferably, they will form a stable duplex at about 15 °C less; more preferably, they will form a stable duplex at about 10 °C less; even more preferably, they will form a stable duplex at about 5 °C less; still more preferably, they will form a stable duplex at about T_m. In another example, if the polypeptide encoded by the polynucleotide is an important part of its functionality, then preferred sequences are those which encode identical or essentially identical polypeptides. Thus, nucleotide differences which cause a conservative amino acid substitution are preferred over those

which cause a non-conservative substitution, nucleotide differences which do not alter the amino acid sequence are more preferred, while identical nucleotides are even more preferred. Insertions or deletions in the polynucleotide that result in insertions or deletions in the polypeptide are preferred over those that result in the down-stream coding region being rendered out of phase; polynucleotide sequences comprising no insertions or deletions are even more preferred. The relative importance of hybridization properties and the encoded polypeptide sequence of a polynucleotide depends on the application of the invention.

A polynucleotide has the same characteristics or is the equivalent of another polynucleotide if both are capable of forming a stable duplex with a particular third polynucleotide under similar conditions of maximal stringency. Preferably, in addition to similar hybridization properties, the polynucleotides also encode essentially identical polypeptides.

"Conserved" residues of a polynucleotide sequence are those residues which occur unaltered in the same position of two or more related sequences being compared. Residues that are relatively conserved are those that are conserved amongst more related sequences than residues appearing elsewhere in the sequences.

"Related" polynucleotides are polynucleotides that share a significant proportion of identical residues.

As used herein, a "degenerate" oligonucleotide sequence is a designed sequence derived from at least two related originating polynucleotide sequences as follows: the residues that are conserved in the originating sequences are preserved in the degenerate sequence, while residues that are not conserved in the originating sequences may be provided as several alternatives in the degenerate sequence. For example, the degenerate sequence AYASA may be designed from originating sequences ATACA and ACAGA, where Y is C or T and S is C or G. Y and S are examples of "ambiguous" residues. A degenerate segment is a segment of a polynucleotide containing a degenerate sequence.

It is understood that a synthetic oligonucleotide comprising a degenerate sequence is actually a mixture of closely related oligonucleotides sharing an identical sequence, except at the ambiguous positions. Such an oligonucleotide is usually synthesized as a mixture of all possibl combinations f nucleotides at the ambigu us

positions. Each of the oligonucleotides in the mixture is referred to as an "alternative form".

A polynucleotide "fragment" or "insert" as used herein generally represents a sub-region of the full-length form, but the entire full-length polynucleotide may also be included.

Different polynucleotides "correspond" to each other if one is ultimately derived from another. For example, messenger RNA corresponds to the gene from which it is transcribed. cDNA corresponds to the RNA from which it has been produced, such as by a reverse transcription reaction, or by chemical synthesis of a DNA based upon knowledge of the RNA sequence. cDNA also corresponds to the gene that encodes the RNA. Polynucleotides also "correspond" to each other if they serve a similar function, such as encoding a related polypeptide, in different species, strains or variants that are being compared.

A "probe" when used in the context of polynucleotide manipulation refers to an oligonucleotide which is provided as a reagent to detect a target potentially present in a sample of interest by hybridizing with the target. Usually, a probe will comprise a label or a means by which a label can be attached, either before or subsequent to the hybridization reaction. Suitable labels include, but are not limited to radioisotopes, fluorochromes, chemiluminescent compounds, dyes, and proteins, including enzymes.

A "primer" is an oligonucleotide, generally with a free 3' -OH group, that binds to a target potentially present in a sample of interest by hybridizing with the target, and thereafter promotes polymerization of a polynucleotide complementary to the target.

Processes of producing replicate copies of the same polynucleotide, such as PCR or gene cloning, are collectively referred to herein as "amplification" or "replication". For example, single or double-stranded DNA may be replicated to form another DNA with the same sequence. RNA may be replicated, for example, by an RNA-directed RNA polymerase, or by reverse-transcribing the DNA and then performing a PCR. In the latter case, the amplified copy of the RNA is a DNA with the identical sequence.

A "polymerase chain reaction" ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using one or more primers, and a catalyst of polymerization, such as a reverse transcriptase or a DNA polymerase, and particularly a thermally stable polymerase enzyme. Generally, a PCR involves reiteratively forming

three steps: "annealing", in which the temperature is adjusted such that oligonucletide primers are permitted to form a duplex with the polynucleotide to be amplified; "elongating", in which the temperature is adjusted such that oligonucleotides that have formed a duplex are elongated with a DNA polymerase, using the polynucleotide to which are formed the duplex as a template; and "melting", in which the temperature is adjusted such that the polynucleotide and elongated oligonucleotides dissociate. The cycle is then repeated until the desired amount of amplified polynucleotide is obtained. Methods for PCR are taught in U.S. Patent Nos. 4,683,195 (Mullis) and 4,683,202 (Mullis et al.).

Elements within a gene include but are not limited to promoter regions, enhancer regions, repressor binding regions, transcription initiation sites, ribosome binding sites, translation initiation sites, protein encoding regions, introns and exons, and termination sites for transcription and translation. An "antisense" copy of a particular polynucleotide refers to a complementary sequence that is capable of hydrogen bonding to the polynucleotide and can therefor, be capable of modulating expression of the polynucleotide. These are DNA, RNA or analogs thereof, including analogs having altered backbones, as described above. The polynucleotide to which the antisense copy binds may be in singe-stranded form or in double-stranded form.

An "antibody" is an immunoglobulin molecule capable of binding an antigen. As used herein, the term encompasses not only intact immunoglobulin molecules, but also anti-idiotypic antibodies, mutants, fragments, fusion proteins, humanized proteins and modifications of the immunoglobulin molecule that comprise an antigen recognition site of the required specificity.

An "antibody complex" is the combination of antibody (as defined above) and its binding partner or ligand.

"Isolated" when referring to a nucleic acid molecule, means separated from other cellular components normally associated with native or wild-type DNA or RNA intracellularly.

An "antisense" copy of a particular polynucleotide refers to a complementary sequence that is capable of hydrogen bonding to the polynucleotide and can therefor, be capable of modulating expression of the polynucleotide. These may be DNA, RNA or analogs thereof, including analogs having altered backbones, as described

above. The polynucleotide to which the antisense copy binds may be in singe-stranded form or in double-stranded form.

As used herein, the term "operatively linked" means that the DNA molecule is positioned relative to the necessary regulation sequences, e.g., a promoter or enhancer, such that a promoter will direct transcription of RNA off the DNA molecule in a stable or transient manner.

"Vector" means a self-replicating nucleic acid molecule that transfers an inserted nucleic acid molecule into and/or between host cells. The term is intended to include vectors that function primarily for insertion of a nucleic acid molecule into a cell, replication vectors that function primarily for the replication of nucleic acid and expression vectors that function for transcription and/or translation of the DNA or RNA. Also intended are vectors that provide more than one of the above functions.

"Host cell" is intended to include any individual cell or cell culture which can be or have been recipients for vectors or the incorporation of nucleic acid molecules and/or proteins. It also is intended to include progeny of a single cell, and the progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation.

A "suitable cell" for the purposes of this invention is one that includes but is not limited to a cell expressing the Fas or TNFR-1 receptor, e.g., a bone marrow cell, endothelial cell, breast carcinoma cell, fibroblast cell, epithelial tumor cell (see Spriggs, D.R. et al. (1988) J. Clin. Inves. 81:455-460) T cell (TCR^+, $CD8^+$ or $CD4^+$ T cells) peripheral blood lymphocyte, colon cell, small intestine cell, ovarian cell, testis cell, prostate cell, thymic cell, spleen cell, kidney cell, liver cell, lung cell, brain cell and monocytes. Because Fas (APO-1/CD95) cell surface "receptor" is a member of the nerve growth factor (NGF)/tumor necrosis factor (TNF) receptor superfamily, any cell having a receptor of this family is intended to be encompassed by the scope of this invention. Fas and TNF receptor expression also has been identified on numerous tissues, see for example Watanabe-Fukunaga et al. (1992) J. Immun. 148:1049-1054 and Owen-Schaub, L.B. et al. (1994) Cancer Res. 54:1580-1586; Dhein et al. (1995) Nature 373:438-441; Brunner et al. (1995) Nature 373:441-444; and Ju et al. (1995) Nature 373:444-448. Assays for identifying additional "suitable" cells sensitive to

induction or activation, e.g., TCR-, TNF- or Fas-related apoptosis, are well known to those of skill in the art. (See for example, Opipairi, et al. J. Biol. Chem. (1992) 267:12424-12427; Yonehara et al. J. Exp. Med. (1989) 169:1747-1756; Dhein et al. (1995) *supra*; Brunner et al. (1995) *supra* and Ju et al. (1995) *supra*). The cells can be mammalian cells or animal cells, such as guinea pig cells, rabbit cells, simian cells, mouse cells, rat cells, or human cells. They can be continuously cultured or isolated from an animal or human. In a separate embodiment of this invention, neurological cells are specifically excluded.

When applied to apoptosis, the terms "preventing" or "inhibiting" are intended to mean a reduction in number of cells dying or a prolongation in the survival time of the cell. They also are intended to mean a diminution in the appearance or a delay in the appearance of morphological and/or biochemical changes normally associated with apoptosis. Accordingly, "augmentation" of apoptotic cell death means an increase in total number of cells dying or reduction of the survival time of the cell. "Augmentation" also means an reduction in the time to the appearance of the morphological and/or biochemical changes normally associated with apoptosis after contacting the cells with the apoptotic agent.

It has been unexpectedly found that the N-terminal truncation of FADD not only abrogates CD95-induced apoptosis; it also abrogates TNFR-1 mediated apoptosis, ceramide generation and activation of the cell death protease Yama. Therefore, as is apparent to those of skill in the art, the utilities of the compositions set forth herein as they relate to the Fas receptor also apply to TNFR-1 mediated cellular functions, such as ceramide generation and activation of the cell death protease Yama. Moreover, the term "FADD" pathway is intended to encompass the pathway mediated by the FADD, which includes, but is not limited to, Fas, TNFR-1, Yama and ceramide generation. A "FADD-associated" function is intended to include cellular functions mediated by the binding of FADD to its intracellular receptors. This includes, but is not limited to, Fas, TNFR-1, CAPP3/CAPP4-mediated cellular functions.

Throughout this application, various publications, patents and published patent applications are referred to by an identifying citation. The disclosures of these publications, patents and published patent applications are hereby incorporated by

reference into this application to more fully describe the state of the art to which this invention pertains.

Proteins and Polypeptides

This invention provides purified proteins designated "FADD" proteins and polypeptides characterized in having the biological or functional ability to modulate a cellular function associated with Fas receptor pathway such as Fas-associated apoptosis. Some of the proteins act to inhibit apoptotic cell death of a suitable cell. Other proteins of this invention act to augment apoptotic cell death. Apoptosis has been equated with programmed cell death (PCD) and can be detected and monitored by a number of morphological and biochemical changes. The methods which are useful to monitor and detect these changes include light microscopy, a measurement between potential and actual tumor doubling times, loss of radiolabeled DNA precursors, measurement of DNA fragmentation, and measurement by FCM. These methods are reviewed Vermes and Haanen, "Apoptosis and Programmed Cell Death in Health and Disease" Adv. in Clin. Chem. (1994) 31:177-246, and the references cited therein. Light microscopy and the measurement of the potential tumor doubling time versus the actual tumor volume doubling time are most applicable in mammalian pathology. "Inhibition" when used in this context, means a reduction in the number of cells undergoing apoptosis or PCD or an increase in survival time or growth rate of a cell or population of treated cells as compared to a control population. "Augmentation" means an increase in the number of cells undergoing apoptosis or PCD or a decrease in survival time or growth rate of a cell or population of treated cells as compared to a control population. A "treated cell" is a cell or a population of cells which have been exposed to the protein or antibody or have inserted therein by any number of methods a nucleic acid molecule of this invention.

As used herein, a FADD protein is intended to include wild-type mammalian and human FADD protein, as well as muteins, analogs, equivalents, and fragments thereof. In some embodiments, the term also includes anti-FADD antibodies and anti-idiotypic antibodies.

In one embodiment of this invention, overexpression of the DNA encoding a FADD protein induces apoptosis. Examples of such proteins include, but are not

limited to full-length wild-type FADD, N-FADD and FADDmt. FADDmt has the amino acid sequence shown in Figure 2A but for an asparagine rather than a valine at position 121.

In a separate embodiment, the biological activity of the FADD protein or its equivalent is inhibitable by *CrmA*. One of skill in the art can determine when and if the biological activity of a protein is inhibitable by *CrmA* using the method disclosed in Tewari et al. (1995) J. Biol. Chem. 270:3255-3260.

A FADD protein can be a purified protein containing 208 amino acids and characterized as having an apparent molecular weight of about 22 to 24 kDa and more particularly about 23.3 kDa, as determined by an SDS polyacrylamide gel (PAGE) under reducing conditions. In one embodiment, these proteins are further characterized as having the ability to bind the intracellular domain of Fas or TNFR-1 and to induce apoptosis in a suitable cell. In one embodiment, the intracellular or cytoplasmic deomain of Fas is intended to encompass any Fas protein or polypeptide containing the death domain shown in Figure 2B. In a separate embodiment, a protein has the amino acid sequence shown in Figure 2A and Sequence ID. No. 2.

Also provided by this invention are polypeptide fragments of the mammalian protein, the human 23.3 kD protein or the proteins having the amino acid sequences shown in Figure 2A. These polypeptide fragments and their equivalents can include any fragment containing the C-terminal half of FADD or a fragment as depicted in Figure 2A from about amino acid 41 to amino acid 208, or from about amino acid 35 to about amino acid 208 and their equivalents. Additional examples include polypeptides comprising the "death domain" of FADD as shown by the underlined region of the amino acid sequence of Figure 2A. These peptides and their equivalents are characterized as being able to bind the cytoplasmic domain of Fas receptor using the *in vitro* binding assay described below. These polypeptides can include, but are not limited to the polypeptides designated NFD-2 (amino acids 42 to 208 of Figure 2A) NFD-3 (amino acids 61 to 208 of Figure 2A) and NFD-4 (amino acids 80 to 208 of Figure 2A).

In a separate embodiment, the fragments contain at least the N-terminal half of the protein, i.e., from about amino acid 1 to about amino acid 125 as shown in Figure 2A. These peptides are characterized by having the ability to induce apoptotic cell

death in a cell without necessarily binding the intracellular domain of the Fas receptor. This activity can be determined using the *in vitro* binding assay described below. These polypeptides can include, but are not limited to the polypeptide designated N-FADD containing from about amino acid 1 to about amino acid 117 as depicted in the sequence provided in Figure 2A and its equivalents.

It is understood that functional equivalents of the protein also shown in Figure 2A, the 23.3 kD purified protein, or the polypeptide fragments thereof, e.g., as shown in Figure 2A and described in Table 1, also are within the scope of this invention. One such equivalent includes the fragments described above having a $V^{121} \rightarrow N^{121}$ alteration. Others include fusion proteins such as those defined herein as clones 8 and 15 or those containing chemical structures other than amino acids which functionally mimic the binding of FADD to the cytoplasmic domain of the Fas receptor ("analogs") or which retain the ability to induce apoptosis with or without binding the cytoplasmic domain of the Fas receptor. An additional example of an analog is a protein or polypeptide containing a distinct protein or polypeptide joined to FADD or fragments thereof, e.g., the GST fusion proteins described herein, the equivalents which vary the primary sequence of protein of this invention from the sequences provided in Figure 2A.

An agent characterized by having the ability to inhibit the binding of FADD to the cytoplasmic domain of Fas receptor is further provided by this invention. Such agents include, but are not limited to, an anti-FADD antibody, a dominant inhibitory fragment of FADD, for example a fragment containing at least the C-terminus of FADD, or a soluble intracellular Fas. "Soluble intracellular Fas" is a polypeptide containing the intracellular death domain portion of the Fas receptor which binds FADD. A "dominant inhibitory fragment" of FADD is intended to include but is not limited to a FADD mutein which irreversibly binds intracellular Fas. Alternative polypeptides containing similar death domains include polypeptides containing sequences shown in Figure 2B.

The proteins and polypeptides of this invention are obtainable by a number of processes well known to those of skill in the art, which include purification, chemical synthesis and recombinant methods. Full length FADD protein can be purified from a Fas^+ cell or tissue lysate using the process described below or by methods such as

immunoprecipitation with anti-FADD antibody, and standard techniques such as gel filtration, ion-exchange, reversed-phase, and affinity chromatography using a FADD fusion protein as shown herein. For such methodology, see for example Deutscher et al., Guide to Protein Purification: Methods in Enzymology (1990) Vol. 182, Academic Press. Accordingly, this invention also provides the processes for obtaining the proteins and polypeptides of this invention as well as the products obtainable and obtained by these processes.

The proteins and polypeptides also can be obtained by chemical synthesis using a commercially available automated peptide synthesizer such as those manufactured by Applied Biosystems, Inc., Model 430A or 431A, Foster City, CA and the amino acid sequence provided in Figure 2A and Table 1. The synthesized protein or polypeptide can be precipitated and further purified, for example by high performance liquid chromatography (HPLC). Accordingly, this invention also provides a process for chemically synthesizing the proteins of this invention by providing the sequence of the protein (e.g., Figure 2A) and reagents, such as amino acids and enzymes and linking together the amino acids in the proper orientation and linear sequence.

Alternatively, the proteins and polypeptides can be obtained by well-known recombinant methods as described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual 2d ed. (Cold Spring Harbor Laboratory (1989)) using the host cell and vector systems described and exemplified below. This invention further provides a process for producing a FADD protein, analog, mutein or fragment thereof, by growing a host cell containing a nucleic acid molecule encoding the mammalian protein, the nucleic acid being operatively linked to a promoter of RNA transcription. The host cell is grown under suitable conditions such that the nucleic acid is transcribed and translated into protein and purifying the protein so produced.

Also provided by this application are the proteins described herein conjugated to a detectable agent for use in the diagnostic methods. For example, detectably labeled proteins and polypeptides containing the C-terminal portion of FADD can be bound to a column and used for the detection and purification of Fas and TNFR-1 receptors. They also are useful as immunogens for the production of anti-FADD antibodies as described below. The proteins and fragments of this invention are useful

in an *in vitro* assay system to screen for agents or drugs which ither inhibit or augment the FADD/Fas- or TNFR-1-receptor pathway and apoptosis and to test possible therapies for disorders and biological processes associated with this pathway, e.g., lps, immunosuppression, depletion of $CD4^+$ T cells, carcinogenesis and embryogenesis.

When used to detect Fas or TNFR-1 or to screen for FADD-related apoptosis-regulating agents, detectably labeled FADD or fragments containing the C-terminal portion of FADD can be bound to an inert solid phase carrier, for example, glass, polystyrene, polyethylene, dextran, nylon, natural and modified celluloses, polyacrylamides, glutathione-agarose beads and agaroses. Those skilled in the art will know of other suitable carriers for this purpose. Accordingly, this invention also provides a composition containing a FADD protein and a method of detecting its receptor in a cell sample by first immobilizing a FADD protein or polypeptide having a binding domain onto a solid support such as glutathione-agarose beads at a suitable concentration. The sample containing or suspected of containing FADD's binding domain (e.g., Fas and TNFR-1) is prepared and contacted with the beads under conditions favoring binding between the receptor and FADD. Alternatively, FADD-CAP3/CAP4 functions can be deduced by screening for agents that inhibit or augment binding of FADD to its downstream partner(s). CAP3/CAP4 is added with agents that may inhibit or augment binding to FADD. This provides a screen for downstream components of the FADD pathway and the agents so identified. Suitable conditions are for example, those set forth in the experimental section described below. The beads are then subjected to conditions to release the complex from the solid support and FADD-Fas complex can then be visualized by autoradiography. The proteins of this invention also can be combined with various liquid phase carriers, such as sterile or aqueous solutions, pharmaceutically acceptable carriers, suspensions and emulsions. Examples of non-aqueous solvents include propyl ethylene glycol, polyethylene glycol and vegetable oils. When used to prepare antibodies, the carriers also can include an adjuvant which is useful to non-specifically augment a specific immune response. A skilled artisan can easily determine whether an adjuvant is required and select one. However, for the purpose of illustration only, suitable

adjuvants include, but are not limited to Freund's Complete and Incomplete, mineral salts and polynucleotides.

This invention also provides a pharmaceutical composition comprising any of a protein, analog, mutein, polypeptide fragment, antibody, antibody fragment or anti-idiotipic antibody of this invention, alone or in combination with each other or other agents, and an acceptable carrier. These compositions are useful for various diagnostic and therapeutic methods.

Nucleic Acids

Nucleic acid molecules and isolated nucleic acid molecules which encode amino acid sequences corresponding to a FADD protein, mutein, analog, FADD polypeptide, antibodies, anti-idiotypic antibody and antibody fragments, as well as complements of these sequences, are further provided by this invention. In addition to the sequences shown in Figure 2A, this invention also provides the anti-sense polynucleotide stand, e.g. antisense RNA. One can obtain an antisense RNA using the sequence provided in Figure 2A and the methodology described in Vander Krol et al. (1988) BioTechniques 6:958.

In one aspect of this invention, the nucleic acid molecule encoding FADD protein or polypeptide is defined to be any of the sequence or parts thereof shown in Figure 2A or which codes for the amino acid sequence also shown in Figure 2A. Also included within the scope of this invention are the DNA or RNA complements of these nucleic acid molecules.

The invention also encompasses nucleic acid molecules which differ from that of the nucleic acid molecules described above, but which produce the same phenotypic effect, such as the allele. These altered, but phenotypically equivalent nucleic acid molecules are referred to "equivalent nucleic acids." This invention also encompasses nucleic acid molecules characterized by changes in non-coding regions that do not alter the phenotype of the polypeptide produced therefrom when compared to the nucleic acid molecule herein. This invention further encompasses nucleic acid molecules which hybridize to the nucleic acid molecules of the subject invention under stringent conditions. Also within the scope of this invention are nucleic acids

having a sequence altered from that shown in Figure 2A but produce a protein having enhanced or diminished biological activity, e.g., FADDmt.

In one embodiment, specifically excluded are the nucleic acid molecules comprising the polynucleotides coding for rFas, hFas and hTNFR-1, the amino acids shown in Figure 2B.

The nucleic acid molecules can be conjugated to a detectable marker, e.g., an enzymatic label or a radioisotope for detection of nucleic acid and/or expression of the gene encoding FADD in a cell. Briefly, this invention further provides a method for detecting a single-stranded nucleic acid molecule encoding an amino acid sequence which is at least a portion of FADD by contacting single-stranded nucleic acid molecules with a labeled, single-stranded nucleic acid molecule (a probe) which is complementary to a single-stranded nucleic acid molecule encoding an amino acid sequence which is at least a portion of FADD protein under conditions permitting hybridization (preferably stringent hybridization conditions) of complementary single-stranded nucleic acid molecules. Hybridized nucleic acid molecules are separated from single-stranded nucleic acid molecules. The hybridized molecules are detected using methods well known to those of skill in the art and set forth, for example, in Sambrook (1989) infra.

The nucleic acid molecules of this invention can be isolated using the technique described in the experimental section described below or replicated using PCR (Perkin-Elmer). For example, the sequence can be chemically replicated using PCR (Perkin-Elmer) which in combination with the synthesis of oligonucleotides, allows easy reproduction of DNA sequences. The PCR technology is the subject matter of United States Patent Nos. 4,683,195, 4,800,159, 4,754,065, and 4,683,202 and described in PCR: The Polymerase Chain Reaction Mullis et al. eds, Birkhauser Press, Boston (1994) and references cited therein. Alternatively, one of skill in the art can use the sequences provided herein and a commercial DNA synthesizer to replicate the DNA. Accordingly, this invention also provides a process for obtaining the polynucleotides of this invention by providing the linear sequence of the polynucleotide, nucleotides, appropriate primer molecules, chemicals such as enzymes and instructions for their replication and chemically replicating or linking the nucleotides in the proper orientation to obtain the polynucleotides. In a separate

embodiment, these polynucleotides are further isolated. Still further, one of skill in the art can insert the nucleic acid into a suitable replication vector and insert the vector into a suitable host cell (a human B cell or BJAB or 293 T cell) for replication and amplification. The DNA so amplified can be isolated from the cell by methods well known to those of skill in the art. A process for obtaining nucleic acid molecules by this method is further provided herein as well as the nucleic acid molecules so obtained.

RNA can be obtained by using the isolated DNA and inserting it into a suitable cell. A suitable cell for this purpose includes but is not limited to a human B cell, BJAB or 293T cell. The DNA can be inserted by any appropriate method, e.g., by the use of an appropriate insertion vector or by electroporation. When the cell replicates and the DNA is transcribed into RNA; the RNA can then be isolated using methods well known to those of skill in the art, for example, as set forth in Sambrook et al. (1989) infra.

The invention further provides the isolated nucleic acid molecule operatively linked to a promoter of RNA transcription, as well as other regulatory sequences for replication and/or transient or stable expression of the DNA or RNA. As used herein, the term "operatively linked" means positioned in such a manner that the promoter will direct transcription of RNA off the DNA molecule. Examples of such promoters are SP6, T4 and T7. In certain embodiments, cell-specific promoters are used for cell-specific expression of the inserted nucleic acid molecule. Vectors which contain a promoter or a promoter/enhancer, with termination codons and selectable marker sequences, as well as a cloning site into which an inserted piece of DNA can be operatively linked to that promoter are well known in the art and commercially available. For general methodology and cloning strategies, see Gene Expression Technology, Goeddel ed., Academic Press, Inc. (1991) and references cited therein and Vectors: Essential Data Series Gacesa and Ramji, eds., John Wiley & Sons, N.Y. (1994), which contains maps, functional properties, commercial suppliers and a reference to GenEMBL accession numbers for various suitable vectors. Preferable, these vectors are capable of transcribing RNA *in vitro* or *in vivo*.

Fragment of the sequences shown in Figure 2A also are encompassed by this invention, preferably at least 10 nucleotides and more preferably having at least 18 nucleotides.

In one embodiment, these fragments are nucleic acid molecules that encode proteins consisting of from about nucleotide 133 to about nucleotide 754, as depicted in Figure 2A. The nucleic acid molecules encode peptides characterized as being able to bind the cytoplasmic domain of Fas or TNFR-1 receptor, using for example, the *in vitro* binding assay described below. Also within the class of nucleic acid molecules are the nucleic acids coding for the peptides designated AU1-NFD-2, AU1-NFD-3, and AU1-NFD-4.

In a separate embodiment, these fragments are nucleic acid molecules that code for amino acids containing at least the N-terminal half of the protein, i.e., from about nucleotide 133 to about nucleotide 501 as shown in Figure 2A. These nucleic acid molecules encode polypeptides characterized as capable of inducing apoptosis in a cell and lacking the ability to bind the intracellular domain of the Fas or TNFR-1 receptor as determined by the *in vitro* assays described below. One example of this class of nucleic acids is a nucleic acid molecule encoding the protein designated N-FADD. This and additional fragments of this invention are useful to code for proteins having diagnostic and therapeutic utilities as described herein as well as probes to identify transcripts of the protein which may or may not be present. These nucleic acid fragments can by prepared, for example, by restriction enzyme digestion of the nucleic acid molecule of Figure 2A and then labeled with a detectable marker such as a radioisotope using well known methods. Alternatively, random fragments can be generated using nick translation of the molecule. For methodology for the preparation and labeling of such fragments, see Sambrook et al., Molecular Cloning: A Laboratory Manual Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989). Nucleic acid fragments of at least 10 nucleotides are useful as hybridization probes or PCP primers. Isolated nucleic acid fragments also are useful to generate novel peptides. These peptides, in turn, are useful as immunogens for the generation of polyclonal and monoclonal antibodies.

As noted above, an isolated nucleic acid m lecule of this invention can be operatively linked to a promoter, either an inducable or non-inducable promoter, of

RNA transcription. These nucleic acid molecules are useful for the recombinant production of FADD and anti-FADD proteins and polypeptides or as vectors for use in gene therapy. Accordingly, this invention also provides a vector (insertion, replication or expression vector) having inserted therein an isolated nucleic acid molecule described above, for example, a viral vector, such as bacteriophage, baculovirus and retrovirus, or cosmids, plasmids, YACS, yeast and other recombinant vectors. Nucleic acid molecules are inserted into vector genomes by methods well known in the art. For example, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules that base pair with each other and which are then joined together with a ligase. Alternatively, synthetic nucleic acid linkers can be ligated to the insert DNA that correspond to a restriction site in the vector DNA, which is then digested with a restriction enzyme that recognizes a particular nucleotide sequence. Additionally, an oligonucleotide containing a termination codon and an appropriate restriction site can be ligated for insertion into a vector containing, for example, some or all of the following: a selectable marker gene, such as neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human cytomegalovirus (CMV) for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and anti-sense RNA.

An additional example of a vector construct of this invention is a bacterial expression vector including a promoter such as the lac promoter and for transcription initiation, the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al. (1989) *supra*). Similarly, a eucaryotic expression vector is a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled using the sequences described herein.

Expression vectors containing these nucleic acids are useful to obtain host vector systems to produce FADD and anti-FADD proteins and polypeptides. It is

implied that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. Suitable expression vectors include viral vectors, including adenoviruses, adeno-associated viruses, retroviruses, cosmids, etc. Adenoviral vectors are particularly useful for introducing genes into tissues *in vivo* because of their high levels of expression and efficient transformation of cells both *in vitro* and *in vivo*. When a nucleic acid is inserted into a suitable host cell, e.g., a procaryotic or a eucaryotic cell and the host cell replicates, the protein can be recombinantly produced. Suitable host cells will depend on the vector and can include mammalian cells, animal cells, human cells, simian cells, insect cells, yeast cells, and bacterial cells constructed using well known methods. See Sambrook et al. (1989) *supra*. In addition to the use of viral vector for insertion of exogenous nucleic acid into cells, the nucleic acid can be inserted into the host cell by methods well known in the art such as transformation for bacterial cells; transfection using calcium phosphate precipitation for mammalian cells; or DEAE-dextran; electroporation; or microinjection. See Sambrook et al. (1989) *supra* for this methodology. Thus, this invention also provides a host cell, e.g. a mammalian cell, an animal cell (rat or mouse), a human cell, or a bacterial cell, containing a nucleic acid molecule encoding FADD, anti-FADD protein or polypeptide or antibody.

Using the host vector system described above, a process of producing and/or obtaining recombinant FADD, analog, mutein, or anti-FADD or active fragments thereof is provided by growing the host cells described herein under suitable conditions such that the nucleic acid encoding the FADD or anti-FADD protein, polypeptide or antibody is expressed. Suitable conditions can be determined using methods well known to those of skill in the art, see for example, Sambrook et al., (1989) *supra*. The recombinant products are then purified from the cellular extract. Accordingly, this invention further provides host cells that contain the nucleic acid molecules of this invention as well as processes for recombinantly producing the proteins, polypeptides and antibodies of this invention by performing the above mentioned steps as well as the products so produced.

A vector containing the nucleic acid encoding FADD, anti-FADD protein, FADD antisense RNA, nucleic acid molecule encoding FADD antisense RNA or antibody also is useful for gene therapy to modulate or regulate TNFR-1 or Fas-

induced cellular functions such as apoptosis and immune disorders mediated by these pathways. The terms "Fas$^+$ or TNFR-1$^+$ cellular function" is intended to mean cellular functions which are affected by the binding of the receptor to its extracellular ligands, i.e., alone or in combination with each other. In some instances, for example in a neoplastic or carcinoma cell, it is desirable to augment apoptotic function to induce apoptosis. This can be achieved by introducing into the cell FADD protein or FADD nucleic acid having this biological activity, e.g., FADDmt or AU1-N-FADD.

In other instances, it is desirable to down-regulate FADD cellular function. This can be accomplished by introducing into the cell an antibody raised against the N-terminus of FADD, anti-FADD antibody, FADD antisense RNA (or the DNA which codes for it) or *CrmA* protein or the nucleic acid molecules coding for these agents. In addition, anti-sense FADD RNA can be used to inhibit production of the FADD protein. This therapy will inhibit or disable intracellular FADD mediated signaling and therefore is a useful therapy where apoptotic cell death is to be avoided, such as in an HIV-infected T cell.

When used for gene therapy *in vivo* or *ex vivo*, a pharmaceutically acceptable vector is preferred, such as a replication-incompetent retroviral vector. Pharmaceutically acceptable vectors containing the nucleic acids of this invention can be further modified for transient or stable expression of the inserted nucleic acid molecule. As used herein, the term "pharmaceutically acceptable vector" includes, but is not limited to, a vector or delivery vehicle having the ability to selectively target and introduce the nucleic acid into dividing cells. An example of such a vector is a "replication-incompetent" vector defined by its inability to produce viral proteins, precluding spread of the vector in the infected host cell. An example of a replication-incompetent retroviral vector is LNL6 (Miller, A.D. et al. (1989) BioTechniques 7:980-990). The methodology of using replication-incompetent retroviruses for retroviral-mediated gene transfer of gene markers is well established (Correll, et al. (1989) PNAS USA 86:8912; Bordignon (1989), PNAS USA 86:8912-52; Culver, K. (1991), PNAS USA 88:3155; and Rill, D.R. (1991) Blood 79(10):2694-700. Clinical investigations have shown that there are few or no adverse effects associated with the viral vectors, see Anderson, (1992) Science 256:808-13.

Compositions containing the nucleic acid molecules of this invention, in isolated form or contained within a vector or host cell are further provided herein. When these compositions are to be used pharmaceutically, they are combined with a pharmaceutically acceptable carrier.

Antibodies

Also provided by this invention is an antibody capable of specifically forming a complex with FADD protein, fragments of FADD such as the N-terminal portion of FADD (anti-N-FADD antibody) anti-FADD antibody or the C-terminal portion of FADD (anti-C-FADD antibody) or a fragment of these antibodies, as well as nucleic acid molecules encoding them. Vectors and host cells containing these nucleic acids also are encompassed by this invention. The term "antibody" includes polyclonal antibodies and monoclonal antibodies. The antibodies include, but are not limited to mouse, rat, rabbit or human antibodies.

As used herein, an "antibody" or "polyclonal antibody" means a protein that is produced in response to immunization with an antigen or receptor and that reacts with the antigen with an effective specificity and affinity for its intended purpose. The term "monoclonal antibody" means an immunoglobulin derived from a single clone of cells. All monoclonal antibodies derived from the clone are chemically and structurally identical, and specific for a single antigenic determinant. The hybridoma cell lines producing the monoclonal antibodies also are within the scope of this invention.

Laboratory methods for producing polyclonal antibodies and monoclonal antibodies, as well as deducing their corresponding nucleic acid sequences, are known in the art, see Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1988) and Sambrook et al. (1989) *supra*. The monoclonal antibodies of this invention can be biologically produced by introducing FADD protein or a fragment thereof into an animal, e.g., a mouse or a rabbit. The antibody producing cells in the animal are isolated and fused with myeloma cells or heteromyeloma cells to produce hybrid cells or hybridomas. Accordingly, the hybridoma cells producing the monoclonal antibodies of this invention also are provided.

Thus, using the FADD protein or fragment thereof, and well known methods, one of skill in the art can produce and screen the hybridoma cells and antibodies of this invention for antibodies having the ability to bind FADD.

If a monoclonal antibody being tested binds with FADD protein, then the antibody being tested and the antibodies provided by the hybridomas of this invention are equivalent. It also is possible to determine without undue experimentation, whether an antibody has the same specificity as the monoclonal antibody of this invention by determining whether the antibody being tested prevents a monoclonal antibody of this invention from binding FADD with which the monoclonal antibody is normally reactive. If the antibody being tested competes with the monoclonal antibody of the invention as shown by a decrease in binding by the monoclonal antibody of this invention, then it is likely that the two antibodies bind to the same or a closely related epitope. Alternatively, one can pre-incubate the monoclonal antibody of this invention with FADD protein with which it is normally reactive, and determine if the monoclonal antibody being tested is inhibited in its ability to bind the antigen. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or a closely related, epitopic specificity as the monoclonal antibody of this invention.

The term "antibody" also is intended to include antibodies of all isotypes. Particular isotypes of a monoclonal antibody can be prepared either directly by selecting from the initial fusion, or prepared secondarily, from a parental hybridoma secreting a monoclonal antibody of different isotype by using the sib selection technique to isolate class switch variants using the procedure described in Steplewski et al. (1985) Proc. Natl. Acad. Sci. 82:8653 or Spira et al. (1984) J. Immunol. Methods 74:307.

This invention also provides biological active fragments of the polyclonal and monoclonal antibodies described above. These "antibody fragments" retain some ability to selectively bind with its antigen or immunogen. Such antibody fragments can include, but are not limited to:

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion with the enzyme papain to yield an intact light chain and a portion of one heavy chain;

(2) Fab', the fragment of an antibody molecule obtained by treating with pepsin, followed by reduction, to yield an intact light chain and a portion f the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3) $F(ab')_2$, the fragment of the antibody that is obtained by treating with the enzyme pepsin without subsequent reduction; $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds;

(4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5) SCA, defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

A specific examples of "biologically active antibody fragment" include the CDR regions of the antibodies. Methods of making these fragments are known in the art, see for example, Harlow and Lane, (1988) *supra*.

The antibodies of this invention also can be modified to create chimeric antibodies and humanized antibodies (Oi, et al. (1986) BioTechniques 4(3):214). Chimeric antibodies are those in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species.

The isolation of other hybridomas secreting monoclonal antibodies with the specificity of the monoclonal antibodies of the invention can also be accomplished by one of ordinary skill in the art by producing anti-idiotypic antibodies (Herlyn, et al. (1986) Science, 232:100). An anti-idiotypic antibody is an antibody which recognizes unique determinants present on the monoclonal antibody produced by the hybridoma of interest. These determinants are located in the hypervariable region of the antibody. It is this region which binds to a given epitope and, thus, it is responsible for the specificity of the antibody. The anti-idiotypic antibody can be prepared by immunizing an animal with the monoclonal antibody of interest. The animal immunized will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants. By using the anti-idiotypic antibodies of the second animal, which are specific for the monoclonal antibodies produced by a single hybridoma which was used to immunize

the second animal, it is now possible to identify other clones with similar idi types as the antibody of the hybridoma used for immunization.

Idiotypic identity between monoclonal antibodies of two hybridomas demonstrates that the two monoclonal antibodies are the same with respect to their recognition of the same epitopic determinant. Thus, by using antibodies to the epitopic determinants on a monoclonal antibody it is possible to identify other hybridomas expressing monoclonal antibodies of the same epitopic specificity.

It is also possible to use the anti-idiotype technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the mirror image of the epitope bound by the first monoclonal antibody. Thus, in this instance, the anti-idiotypic monoclonal antibody could be used for immunization for production of these antibodies.

As used in this invention, the term "epitope" is meant to include any determinant having specific affinity for the monoclonal antibodies of the invention. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

Also encompassed by this invention are proteins or polypeptides that have been recombinantly produced, biochemically synthesized, chemically synthesized or chemically modified, that retain the ability to bind FADD, the intracellular binding domain of the Fas receptor, or a fragment thereof, as the corresponding native polyclonal or monoclonal antibody.

The antibodies of this invention can be linked to a detectable agent or a hapten. The complex is useful to detect the Fas receptor or FADD protein or fragments in a sample or detect agents which interfere with FADD-Fas receptor binding, using standard immunochemical techniques such as immunohistochemistry as described by Harlow and Lane (1988) *supra.* Examples of types of immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or an indirect format. Examples of such immunoassays are the enzyme linked immunoassay (ELISA) radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of using the monoclonal

antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

Another technique which may also result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin, which reacts avidin, or dinitropherryl, pyridoxal, and fluorescein, which can react with specific anti-hapten antibodies. See Harlow and Lane (1988) *supra.*

The monoclonal antibodies of the invention can be bound to many different carriers. Thus, this invention also provides compositions containing the antibodies and another substance, active or inert. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, and bioluminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the monoclonal antibody, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the monoclonal antibody of the invention can be done using standard techniques common to those of ordinary skill in the art.

For purposes of the invention, FADD may be detected by the monoclonal antibodies of the invention when present in biological fluids and tissues. Any sample of cells or tissue lysate containing a detectable amount of FADD can be used.

Compositions containing the antibodies, fragments thereof or cell lines which produce the antibodies, are encompassed by this invention. When these compositions

are to be used pharmaceutically, they are combined with a pharmaceutically acceptable carrier.

Compositions

This invention also provides compositions containing any of the above-mentioned proteins, muteins, polypeptides, nucleic acid molecules, vectors, host cells antibodies and fragments thereof, and an acceptable solid or liquid carrier. When the compositions are used pharmaceutically, they are combined with a "pharmaceutically acceptable carrier" for diagnostic and therapeutic use. These compositions also can be used for the preparation of medicaments for the diagnosis and treatment of pathologies associated with FADD pathways, e.g., Fas and TNFR-1 receptor pathways.

Industrial Applicability

The compositions described above provide the components for an assay to screen for agents and pharmaceutical compounds which are agonists or antagonists of the FADD pathway in suitable cells and which can modulate a cellular function regulated by this receptor pathway such as apoptotic cell death. In one embodiment, these cells constitutively and inducibly express receptors for either or both of the cytokine tumor necrosis factor (TNF) or the cell death transducing receptor Fas or TCR and which have been activated by their respective ligand. Recently, three separate groups have reported that Fas-induced apoptosis is involved in T cell death. Specifically, one group has shown that the Fas receptor, which can transduce a potent apoptotic signal when ligated, is rapidly expressed following activation on T cell hybridomas. It was suggested that the Fas receptor-ligand interaction induces cell death in a cell-autonomous manner. See Dhein et al. (1995) Nature 373:438-441; Brunner et al. (1995) Nature 373:441-444; and Ju et al. (1995) Nature 373:444-448.

For the purpose of illustration only, examples of suitable cells are T lymphocytes (T cells) (e.g., TCR^+, $CD4^+$ and $CD8^+$ T cells) leukocytes and mixed leukocyte cultures (MLC), B lymphoma cells (e.g., A202J (ATCC)), peripheral blood lymphocytes, colon cells, small intestine cells, ovarian cells, testis cells, prostate cells, thymic cells, spleen cells, kidney cells, liver cells. neoplastic cells, carcinoma cells,

lung cells or brain cells, each from a mammalian species, e.g., mouse, rat, simian guinea pig, or human.

As provided in more detail below, the proteins and fragments thereof are useful in a cell-free and cellular *in vitro* assay system to screen for agents and pharmaceutical compounds which either inhibit or augment the FADD pathway and apoptosis and to test possible therapies for disorders associated with this pathway, e.g., lps, immunosuppression, depletion of $CD4^+$ T cells, carcinogenesis and embryogenesis.

To perform the cell free screen, an effective amount of the cytoplasmic domain of a receptor known to initiate this pathway, e.g. Fas or TNFR-1, is first bound to a solid support (for example, glass, polystyrene, polyethylene, dextran, nylon, natural and modified celluloses, polyacrylamides, glutathione-agarose beads and agaroses in a suitable concentration. Those skilled in the art will know of other suitable carriers for this purpose. A diagnostically effective amount of detectably labeled agent to be tested is contacted under suitable conditions which favor binding of the cytoplasmic domain of the receptor to FADD. An effective amount of FADD polypeptide containing at least the cytoplasmic binding domain of the receptor is now contacted to the solid support under conditions favoring binding of the cytoplasmic domain receptor to FADD. Procedures for the detecting of complex are then performed, for example, was a complex formed between the receptor and FADD to form a receptor-FADD complex or did the agent bind to the receptor or FADD? If an agent binds tightly to FADD, it is a candidate for preventing or inhibiting FADD induced cellular responses such as apoptosis so that FADD cannot interact with its downstream component.

Alternatively, the method can be performed by: a) providing an effective amount of the cytoplasmic domain of the receptor bound to a solid support; b) contacting an effective amount of FADD polypeptide containing at least the C-terminal portion of protein to the solid support of step a) under conditions favoring binding of the cytoplasmic domain receptor to FADD; c) contacting an effective amount of detectably labeled agent to be screened with the receptor bound support of step b) under conditions favoring binding of the cytoplasmic domain to the receptor to FADD; d) detecting the presence of any complex f rmed either between the receptor

and FADD to form a receptor-FADD complex or between the agent and receptor. If an agent binds tightly to FADD, it is a candidate for preventing or inhibiting FADD induced cellular responses such as apoptosis, by binding to FADD so that FADD can not interact with its downstream component.

A further alternative method is provided. In this method, an effective amount of a FADD fragment containing at least the N-terminal portion of FADD (N-FADD) is bound to a solid support. An effective amount of the agent to be tested is contacted with the support under conditions favoring binding of the N-terminal portion of FADD to its ligand (e.g., anti-N-FADD antibody). Either the agent or N-FADD can be detectably labeled. The presence of complex, if any is then detected. If an agent binds to N-FADD it is a candidate for preventing or inhibiting FADD induced cellular responses such as apoptosis by binding to the N-terminal portion of FADD so that FADD can not interact with its downstream component in the FADD pathway.

Also encompassed by this invention are the agents detected by these methods, the nucleic acid molecules encoding them and the use of these agents and nucleic acid molecules in the therapeutic methods described herein. As is apparent to those of skill in the art, the above compositions can be combined with instructions for use to provide a kit for a commercially available screen.

The above methods allow one also to screen for drugs having similar or enhanced ability to prevent or inhibit apoptosis as compared to FADDmt or *CrmA*, for example.

In the cellular *in vitro* method, suitable cell cultures or tissue cultures are provided. A suitable cell culture for this purpose is one having either a cell surface receptor that mediates apoptosis such as a TCR, the TNF receptor or the Fas receptor. The cells are cultured under conditions (temperature, growth or culture medium and gas (CO_2)) and for an appropriate amount of time to attain exponential proliferation without density dependent constraints. The cells are then exposed to preliminary conditions necessary for apoptosis, for example an effective amount of an inducing agent, e.g., a TCR ligand, HIV, SIV, TNF, or a Fas ligand such as an anti-Fas antibody is added to the culture. Anti-Fas and anti-TNFR-1 antibodies and mitogens (ConA) are well known to those of skill in the art. (Itoh, N. et al. (1991) Cell 66:233-243 and Yonehara et al. (1989) J. Exp. Med. (1989) 169:1747-1756). These cells are

now "induced" to apoptosis. Alternatively, the cells can be contacted with the inducing agent after transfection with the FADD nucleic acid and agent. The cells are again cultured under suitable temperature and time conditions. An effective amount of an agent which induces apoptosis in this system is added to the cell culture. For example, an effective amount of a nucleic acid molecule encoding FADDmt or N-FADD, or wild-type FADD is contacted with the cell or tissue culture so as to insert the nucleic acid. In one embodiment, an effective amount is an amount so that overexpression of the inserted nucleic acid molecule can occur. Alternatively, an effective amount of the polypeptide or protein products are added to the cell culture. The cells are again cultured for expression of the inserted nucleic acid molecule. An effective amount of the agent to be tested is then added to the cell or tissue culture in varying concentrations.

Because the activity of FADDmt and FADD is inhibitable by *CrmA*, a separate culture of cells which can act as a comparison is cultured under identical conditions as described above, except that *CrmA* nucleic acid is added to the culture rather than the agent. The *CrmA* nucleic acid or protein is added the culture in an effective amount and the cells are cultured under suitable temperature and time conditions to inhibit apoptosis. The *CrmA* nucleic acid or protein can be added prior to, simultaneously with, or after, the inducing agent.

It also is desirable to maintain an additional separate cell cultures; one which does not receive an inducing agent to determine background release and another which does not receive the agent to be tested.

Each of the samples of cells is then assayed for apoptotic activity using methods well known to those of skill in the art and described herein.

An example of the above method comprises providing a T cell hybridoma cell line such as Jurkat that can be stably transfected with the FADD nucleic acid and *CrmA* nucleic acid. Clonal cell lines of each are derived. Transfection of the Jurkat cell by electroporation can be performed as described in Dixit et al. J. Biol. Chem. (1993) 263:5032-5039. *CrmA*-expressing and agent contact control cells are ^{51}Cr-labeled and plated (5×10^5 /ml) on untreated or anti-CD3 (available from the cell line 145-2C11 (ATCC)) treated tissue culture plastic plates. Cells cultured on uncoated cells are used to determine background release. The percentage cell death will be

patient or an animal such as a mouse, a guinea pig, a simian, a rabbit or a rat. Alternatively, it can be directly infused into the cell by microinjection.

When the animal is an experimental animal such as a simian (using SIV), this method provides a powerful assay to screen for new drugs that may be used alone or in combination with this invention to ameliorate or reduce the symptoms and opportunistic infections associated with HIV infection or AIDS. When the animal is an experimental animal such as a mouse, this method provides a powerful assay to screen for new drugs that may be used alone or in combination with this invention to ameliorate or reduce the symptoms and infections associated with Fas-related disfunction such as $CD4^+$ T cell depletion or hyperproliferative disorders such as cancer.

Because this invention provides compositions and methods to increase survival time and/or survival rate of a cell or population of cells which, absent the use of the method, would normally be expected to die, also provided by this invention are methods to prevent or treat diseases or pathological conditions associated the FADD-receptor pathway such as with unwanted apoptotic cell death in a subject.

For example, the compositions provided herein are useful to modulate the FADD receptor pathway and cellular functions associated with this pathway by preventing or inhibiting FADD regulated cellular functions such as apoptosis or growth and differentiation of cells. As noted above, the term "FADD mediated or modulated cellular function" is to include any cellular response or function which has been linked to the binding of FADD to its cellular targets such as Fas, TNFR-1 or CAPP3/CAPP4. Apoptotic cell death is one such response.

Methods of modulating FADD cellular functions such as apoptotic cell death are provided herein. These methods comprise the steps of administering to the subject, such as an animal or human, an effective amount of a FADD nucleic acid, antibody or protein. When the cellular function is augmentation of apoptotic cell death, an effective amount of a nucleic acid molecule coding for FADDmt or wild-type FADD or their protein products can be administered to the subject. In one embodiment, an effective amount is an amount which allows for overexpression of the inserted nucleic acid molecule. When the cellular function is inhibition or prevention of apoptotic cell death, an effective amount of a nucleic acid molecule coding for N-

FADD or anti-N-FADD antibody or their protein products are administered to the subject. Alternatively, an effective amount of DNA encoding FADD antisense RNA or the RNA is administered to the subject.

When practiced *in vivo*, the compositions and methods are particularly useful for modulating or regulating FADD functions in a subject or an individual suffering from or predisposed to suffer from receptor-related disfunction or for maintaining T cell viability and function in a subject or an individual suffering from or predisposed to suffer from abnormal lymphocyte death, e.g. CD4+ T cell depletion associated with HIV infection. When the method is practiced *in vivo* in a human patient, it is unnecessary to provide the inducing agent since it is provided by the patient's immune system. When the method is practiced *in vivo*, the carrying vector, polypeptide, polypeptide equivalent, or expression vector can be added to a pharmaceutically acceptable carrier and systemically administered to the subject, such as a human patient or an animal such as a mouse, a guinea pig, a simian, a rabbit or a rat. Alternatively, it can be directly infused into the cell by microinjection or localized administration into a tumor. A fusion protein also can be constructed comprising the T-cell specific ligand for targeting to a T cell. Such T cell specific ligands include, but are not limited to anti-CD3, anti-CD4, anti-CD28 and anti-IL-1 antibody protein.

This invention also is particularly useful to ward off lymphocyte death or immunosuppression in AIDS patients. By preventing or inhibiting apoptosis, not only is cell death prevented but functionality, e.g., immuno-proliferative capacity, is restored to the cell and a responsive immune system is retained or regained. Accordingly, the compositions and methods of this invention are suitably combined with compositions and methods which prevent or inhibit HIV infectivity and replication.

The method also can be practiced *ex vivo* using a modification of the method described in Lum et al. (1993) Bone Marrow Transplantation 12:565-571 or a modification of the method described in U.S. Patent No. 5,399,346. Generally, a sample of cells such as bone marrow cells or MLC can be removed from a subject or animal using methods well known to those of skill in the art. An effective amount of FADD or anti-FADD nucleic acid is added to the cells and the cells are cultured under conditions that favor internalization of the nucleic acid by the cells. The transformed

cells are then returned or reintroduced to the same subject or animal (autologous) or one of the same species (allogeneic) in an effective amount and in combination with appropriate pharmaceutical compositions and carriers.

Alternatively, fresh peripheral blood mononuclear cells (MNCS) isolated from the mammal or patient are separated from the red cells and neutrophils by Ficoll-Hypaque density gradient centrifugation. The MNCs are then washed, counted and cultured at approximately 1 X 10^6 cells/well in a 24 well tissue culture plates in AIM-V which consists of AIM-V (GIBCO) with 2mM glutamine, 50 U/mi penicillin, 50 Tg/ml streptomycin, 2.5 Tg/ml Fungizone and 25-1,000U/ml of IL-2 (Cetus). The cells are cultured at 37°C in a humidified incubator with 5%CO.

After the T cells have begun to proliferate, an appropriate insertion vector containing a FADD nucleic acid molecule is contacted with the cells to insert FADD nucleic acid into the proliferating cells. Multiple transfection of the cells may be necessary. The cells are maintained for an additional 2 to 7 days with fresh medium and under conditions to return the cells to exponential growth. Approximately 0.1 to 2.5 X 10^{10} T cells (or 80% of the total culture) are infused into the mammal or patient and the remaining cells can be cyropreserved for future infusions. A sample of the cells also can be removed for Southern analysis of insertion of the FADD nucleic acid molecule and its expression using northern analysis.

As used herein, the term "administering" for *in vivo* and *ex vivo* purposes means providing the subject with an effective amount of the nucleic acid molecule or polypeptide effective to modulate the FADD cellular function, e.g., to prevent, inhibit or augment apoptosis of the target cell. Methods of administering pharmaceutical compositions are well known to those of skill in the art and include, but are not limited to, microinjection, intravenous or parenteral administration. The compositions are intended for topical, oral, or local administration as well as intravenously, subcutaneously, or intramuscularly. Administration can be effected continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage f administration are well known to those of skill in the art and will vary with the vector used for therapy, the polypeptide or protein used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and

pattern being selected by the treating physician. For example, the compositions can be administered prior to a subject already suffering from a disease or condition that is linked to apoptosis. In this situation, an effective "therapeutic amount" of the composition is administered to prevent or at least partially arrest apoptosis and the accompanying pathology such as immunosuppression in HIV infected individuals.

However, the compositions can be administered to subjects or individuals susceptible to or at risk of developing apoptosis-related disease to prevent pathological cell death. In one embodiment, the composition can be administered to a subject susceptible to HIV-related lymphocyte disfunction to maintain lymphocyte cell function and viability. In these embodiments, a "prophylactically effective amount" of the composition is administered to maintain cellular viability and function at a level near to the pre-infection level.

It should be understood that by preventing or inhibiting unwanted cell death in a subject or individual, the compositions and methods of this invention also provide methods for treating, preventing or ameliorating the symptoms associated with a disease characterized by apoptosis of cells. Such diseases include but are not limited to AIDS, acute and chronic inflammatory disease, leukemia, myocardial infarction, stroke, traumatic brain injury, neural and muscular degenerative diseases, aging, tumor induced-cachexia and hair loss.

This invention also provides vector and protein compositions useful for the preparation of medicaments which can be used for preventing or inhibiting apoptosis, maintaining cellular function and viability in a suitable cell or for the treatment of a disease characterized by the unwanted death of target cells.

It also is intended that the compositions and methods of this invention be combined with other suitable compositions and therapies such as the use of *CrmA* and antidiotypic TCR antibodies.

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and the following examples are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

Experiment I

Isolation of FADD

The following yeast two-hybrid system was used and constructed as follows. The cytoplasmic domains of Fas, Fas-FD8, TNRF-1, ΔTNFR-1, CD40, and CD28 were obtained by PCR and cloned in-frame, as confirmed by sequencing, into the GAL4 DNA binding domain (GAL4bd) vector pAS1CYH2. Full-length A20 and B94 were similarly cloned into the bait vector. GAL4bd-Fas was cotransformed with a prey plasmid containing a human B-cell cDNA expression library fused to the GAL4 activation domain (GAL4ad) in the pACT plasmid. A more detailed account of the plasmids used in the procedure for the yeast two-hybrid system can be found in Hu et al. (1994) J. Biol. Chem. 269:30069-30072.

The yeast two-hybrid system was used to screen for proteins that interact with the cytoplasmic domain of Fas. An expression vector was constructed by fusing the GAL4 DNA-binding domain to the cytoplasmic tail of the human Fas antigen (GAL4bd-Fas). This bait plasmid was cotransformed in yeast with a prey plasmid containing a human B-cell cDNA expression library fused to the GAL4-activation domain. Seventeen positive clones were obtained from 2×10^6 transformants screened. To determine the specificity of interaction, plasmids containing the activation domain fusion proteins were recovered from the putative positive clones and cotransformed with GAL4bd-Fas and control heterologous baits. Two clones (8 and 15) were found to interact with the GAL4 DNA-binding domain fusion protein containing the cytoplasmic domain of wild-type Fas and not the functionally inactive deletion mutant, Fas-FD8 (Itoh et al. (1993) *supra*) or the indicated heterologous baits (Figure 1).

Isolation of the sequence

Double-stranded plasmid template was sequenced on both strands by the dideoxy chain termination method using modified T7 DNA polymerase (Sequenase, U.S. Biochemical Corp.). Manual sequencing was confirmed by subsequent automated sequencing. Network BLAST searches were conducted using the NCBI-

nline service. Sequences were compared using the MogAlign (DNASTAR) software.

A random-primed cDNA library was constructed in the pcDNA1 vector (Invitrogen) from TNF/cycloheximide treated human umbilical vein endothelial cell poly(A)$^+$ RNA. 5 x 10^5 colonies were screened with a ^{32}P random-labeled XhoI restriction fragment of the yeast prey plasmid encoding GAL4ad-FADD (clone 15) using standard techniques (Sambrook et al. (1989) *supra*).

As noted in Experiment I, clones 8 and 15 isolated by the yeast two-hybrid screen were found to contain overlapping sequence fused to the GAL4 activation domain in the same reading frame. To obtain a full-length coding sequence, a human umbilical vein endothelial cell (HUVEC) library was screened with a cDNA insert obtained from clone 15. Two independent clones yielded a 1.6 kb cDNA containing an open reading frame that begins with an initiator methionine conforming to Kozak's consensus (Kozak, M. (1989) J. Cell. Biol. 108:229-241) and that ends 625 nucleotides later at an Opal codon. Given the presence of an in-frame stop codon 130 base pairs upstream of the initiator methionine and the size of the transcript (~1.6 kb; Figure 3), Figure 2A represents the full-length coding sequence. This gene encodes a novel protein of 208 amino acids with a predicted molecular weight of 23.3 kDa, designated FADD.

A BLAST search revealed that residues 111-170 of FADD matched residues 233-292 of rat Fas antigen (rFas, p=0.0012) and shared 27% identity (51% of the amino acids were conserved). This region in the cytoplasmic domain of rFas corresponds to the death domain, a region of homology shared by both Fas and TNFR-1 that signals cell death (Tartaglia et al. (1993) *Supra*, and Itoh et al. (1993) *supra*.)

Dependent upon the alignment and boundaries selected, the death domains of FADD, Fas, and TNFR-1 share 25-30% identity (Figure 2B). When conservative amino acid substitutions are included, the homologies approach 50%. These numbers are consistent with those previously reported for the death domain homology between TNFR-1 and Fas (Tartaglia et al. (1993) *supra*; Itoh et al. (1993) *supra*). Interestingly, V^{121} of FADD is aligned and conserved with V^{238} of Fas, which when altered to an asparagine, abolishes the cell killing activity of Fas and in mice, is

responsible for the lymphoproliferation (*Lpr*) phenotype (Watanabe-Fukunaga et al. (1992) *supra*.) A corresponding inactivating mutation also exists in TNFR-1, $L^{351} \rightarrow N^{351}$ (Tartaglia et al. (1993) *supra*.)

Northern Blot analysis of tissues

Adult and fetal human multiple tissue Northern blots (CLONTECH) were hybridized, according to the manufacturer's instructions using radiolabeled cDNA insert obtained from an XhoI digestion of the yeast prey plasmid encoding GAL4ad-FADD (clone 15).

Northern blot analysis revealed that FADD is constitutively expressed in a wide array of fetal and adult human tissues (Figures 3A and 3B). The mRNA transcript is approximately 1.6 kb, consistent with the size of the cDNA clones isolated from the HUVEC library.

***In Vivo* Association of FADD and Fas**

FADD was cloned into pcDNA3 (Invitrogen) in which an HA-epitope tag (YPYDVPDYA-SEQ ID NO:7) had previously been placed downstream of the cytomegalovirus promoter/enhancer (pcDNA3 HA-FADD). In addition, an AU1-epitope (DTYRYI-SEQ ID NO:8) tagged FADD was made with PCR primers encoding the epitope and using the FADD cDNA as template (pcDNA3 AU1-FADD). FLAG (DYKDDDDK-SEQ ID NO:9)-tagged constructs of Fas and mutants were also made in pcDNA3 using full-length Fas as a template. The 5' FLAG PCR primer was engineered to encode a FLAG epitope 5 amino acids downstream of the putative signal sequence site of Fas and is as follows: AAG CCT GGT ACC ATG CTG GGC ATC TGG ACC CTC CTA CCT CTG GTT CTT ACG TCT GTT GCT AGA TTA TCG TCC AAA GAC TAC AAG GAC GAC GAT GAC AAG AGT GTT AAT GCC CAA GTC (SEQ ID NO:10). The amplified products were then cloned into the KpnI/XhoI site of pcDNA3. pcDNA3 AU1-FADDmt and pcDNA3 FLAG-Fas-LPR were made by site-directed mutagenesis using a two-step PCR protocol as described in Higuchi, R. et al. (1988) Nucleic Acids Res. 16:7351-7367. The $V^{121} \rightarrow N^{121}$ and $V^{238} \rightarrow N^{238}$ mutations, respectively, were confirmed by sequence analysis.

GST Fusion Protein Expression and *In Vitro* Binding Assay

The cytoplasmic domains of Fas, Fas-FD5, Fas-FD8, and TNFR-1 were amplified by PCR using appropriate templates and primers and cloned in-frame into pGSTag using the method disclosed in Ron, D. et al. (1992) Biotechniques 13:866–869. Fas-LPft was made by site-directed mutagenesis using a two-step PCR protocol (Higuchi et al. (1988) *supra*) and cloned into pGSTag. The $V^{238} \rightarrow N^{238}$ mutation was confirmed by sequence analysis. The pGSTag constructs were then transformed into the *E. coli* strain BL21(DE3)pLysS (Studier (1991) J. Mol. Biol. 219:37-44). GST and GST fusions were prepared using published procedures (Studier, (1991) *supra*) and the recombinant proteins immobilized onto glutathione-agarose beads as described in Harper, J.W. et al. (1993) Cell 75:805-816.

Labeled FADD was prepared by *in vitro* transcription/translation using TNT T7 coupled reticulocyte lysate system from Promega according to the manufacturer's instructions, using pcDNA3 HA-FADD as template.

Following translation, equal amounts of total ^{35}S-labeled reticulocyte lysate were diluted into 150 µl GST binding buffer (50 mM Tris, pH 7.6, 120 mM NaCl, 1% Brij) and incubated for 2 hrs. at 4°C with the various GST fusion proteins complexed to beads, following which the beads were pelleted by pulse centrifugation, washed 3 times in GST buffer, boiled in SDS-sample buffer and resolved on a 10% SDS-acrylamide gel. Bound proteins were visualized following autoradiography at -80°C.

Lysates of FADD or FADDmt-transfected 293T cells were processed as above except that the GST binding buffer also had 10% glycerol and a protease inhibitor cocktail. For some experiments, the complexed GST beads were dissociated by boiling in PBS + 1% SDS, diluted tenfold in PBS containing 1% deoxycholate and subsequently subjected to immunoprecipitation analysis.

Transfection, Metabolic Labeling and Immunoprecipitation Analysis

These techniques were performed as described in O'Rourke, K.M. et al. (1992) J. Biol. Chem. 267:24921-24924. For re-immunoprecipitation analysis, the initial immune complex was dissociated by boiling in PBS + 1% SDS, diluted tenfold in PBS containing 1% deoxycholate and subjected to a second round of immunoprecipitation analysis.

To confirm the interaction observed in yeast, radiolabeled *in vitro* translated FADD was precipitated with various GST fusion proteins immobilized on glutathione-Sepharose beads (Figure 4A, B). As predicted, FADD specifically associated with GST-Fas but not GST, GST-Fas-FD8, or GST-Fas-LPR, which contains the cytoplasmic domain of the functionally inactive point mutant of Fas (Itoh et al. (1993) *supra*). A very weak interaction was observed between FADD and TNFR-1. Interestingly, relative to its association with GST-Fas, FADD strongly interacted with GST-Fas-FD5, which is a 15 amino acid C-terminal deletion mutant of Fas possessing enhanced killing activity (Itoh et al. (1993) *supra*). Similar results were obtained when detergent lysates of 293T cells expressing FADD were precipitated with the various GST fusion proteins (Figure 4C).

Functional Assay and Immunocytochemistry

Stable *CrmA* and vector transfectants (BJAB) were described previously (Tewari et al. (1995) *supra*). For transient transfections, 5 x 10^6 cells were electroporated at 220V, 960 μF in 0.4 cm cuvettes (Bio-Rad) using 20 μg of pCMV β-galactosidase ± 30 μg of pcDNA3 AU-FADD. After 12 hours, cells were cytocentrifuged, fixed with 1% paraformaldehyde, permeabilized with 0.1% Trigon/PBS, blocked with horse serum, and incubated with rabbit anti-β-galactosidase (1:200 dilution, Cappel) for 1 hour. The cells were subsequently washed with PBS, incubated with biotinylated anti-rabbit antibody (1:200 dilution, Vector Laboratories) for 20 min., washed with PBS, and incubated with Avidin-FITC (1:100 dilution, Vector Laboratories) for 20 min. The nuclei were stained with a 10 μg/ml solution of propidium iodide (Sigma) for 10 minutes. Cells were visualized by fluorescence microscopy using a FITC range barrier filter cube. For graphical data, at least 100 β-galactosidase positive cells were counted for each transfection (n=3) and designated as apoptotic or non-apoptotic. Immunostaining for AU1-FADD was done as above except that cells were fixed in 100% methanol at -20°C for 10 min., the primary antibody was against the AU1 epitope (1:50 dilution, Babco) and the secondary antibody was a FITC conjugated anti-mouse Ab (Sigma).

Coimmunoprecipitation of FADD and Fas

To demonstrate the interaction of FADD and Fas *in vivo*, 293T cells were transiently transfected with HA-epitope tagged FADD (HA-FADD) and FLAG-epitope tagged Fas (FLAG-Fas) and mutants (Figures 5A through 5C). Expression of the FLAG-tagged constructs was shown by immunoprecipitation with an anti-FLAG (α-FLAG) antibody (Figure 5B). Likewise, immunoprecipitation with anti-HA (α-HA) antibody showed expression of HA-FADD, and as expected, FLAG-Fas and FLAG-Fas-FD5 individually coprecipitated, while the functionally inactive mutants, FLAG-Fas-FD8 and FLAG-Fas-LPR did not (Figure 5C). The α-HA immunoprecipitates were dissociated and subjected to a second round of immunoprecipitation with α-FLAG antibody. Consistent with results of the primary immunoprecipitation (with α-HA), a double immunoprecipitation with α-HA followed by α-FLAG, confirmed the presence of FLAG-Fas and FLAG-Fas-FD5 in the original immune complexes (Figure 5D).

The Death Domain of FADD Interacts With The Death Domain of Fas

Previous studies have reported that the death domains of TNFR-1 and Fas self-associate. The two clones (8 and 15) isolated in the two-hybrid screen described above (using the cytoplasmic domain of Fas as bait) did not contain various portions of the N-terminus of wild-type FADD. The shortest of the two, clone 8, is missing the N-terminal 40 amino acids, suggesting that the C-terminal half of FADD, which contains the death domain, is interacting with the cytoplasmic tail of Fas. More specifically, our results show that FADD interacts with death domain of Fas, since it fails to associate with Fas-LPR and Fas-FD8, a point mutant and deletion mutant, respectively, of the Fas death domain.

Thus, it is reasonable to propose that the death domain of FADD is interacting with its homologous counterpart in Fas. To test this hypothesis, a point mutant of FADD (FADDmt) was engineered in which V^{121} is altered to an asparagine. This mutation corresponds to the inactivating *Lpr* mutation ($V^{238} \rightarrow N^{238}$) of Fas and the $L^{351} \rightarrow N^{351}$ mutation of TNFR-1. 293T cells were transiently transfected with expression constructs containing AU1-epitope tagged FADD (AU1-FADD) and AU1-

FADDmt. Detergent lysates were prepared and subsequently precipitated with GST, GST-Fas and GST-Fas-LPR immobilized on glutathione-Sepharose beads (Figure 6). As predicted, AU1-FADD bound GST-Fas and not GST or GST-Fas-LPR, while in contrast, AU1-FADDmt failed to bind any of the GST fusions. Taken together, these results show that a death domain to death domain interaction is responsible for the association of FADD and Fas.

Overexpression of FADD Initiates Apoptosis Which Is Suppressed by CrmA.

To study the functional role of FADD the B-cell lymphoma cell line, BJAB was chosen. This is an ideal cell system to study proteins involved in Fas signal transduction because BJAB cells are exquisitely sensitive to anti-Fas antibody induced cell death in the absence of protein synthesis inhibitors (Tewari et al. (1995) *supra*). Two well characterized clonal cell lines of BJAB were used in this study: one expresses *CrmA*, which has been shown to potently block Fas-mediated cell death, while the other is a corresponding vector control cell line (Tewari et al. (1995) *supra*). To help identify transiently transfected cells, the plasmid was co-transfected with an expression construct encoding β-galactosidase (pCMV β-gal). As expected, over 90% of the cells that expressed β-galactosidase also expressed the protein of interest as confirmed by immunostaining.

CrmA-expressing and vector control BJAB cell lines were transfected with the pCMV β-gal reporter in the presence or absence of an equimolar amount of an expression construct encoding AU1-epitope tagged 60FADD (pcDNA3 AU1-FADD). As expected, expression of β-galactosidase alone in both the *CrmA* and vector clones did not induce apoptotic cell death as assessed by propidium iodide staining of nuclei of β-galactosidase positive cells (Figure 7A, upper panels). In contrast, however, the vector control cell line co-transfected with pCMV β-gal and pcDNA3 AU1-FADD exhibited prominent apoptotic morphology including chromatin condensation and cellular shrinkage (Figure 7A, lower left panel). More importantly, FADD-induced apoptosis, like Fas-induced apoptosis, was inhibited in the *CrmA*-expressing line (Figure 7A, lower right panel). A graphical representation of this data is shown in Figure 7B. In the vector control lines, over 90% of the transfected cells expressing FADD were apoptotic while less than 10% exhibited similar morphology in the

corresponding *CrmA*-expressing lines. As a control, expression of AU1-TRAF1 and HA-CD40bp revealed less than 10% apoptotic morphology in either the *CrmA* or vector cell lines. Immunostaining for AU1-FADD with an anti-AU1 antibody is shown in Figure 7C.

A second cell line, MCF7 cells, a breast carcinoma cell line which expresses Fas and is sensitive to Fas-induced killing in the presence of the protein synthesis inhibitor cycloheximide (Tewari et al. (1995) *supra*). Two stably transfected and previously characterized clonal cell lines of MCF7 were used in our study: one expresses *CrmA*, a viral serpin inhibitor which has been shown to potently block Fas-mediated cell death (Tewari et al. (1995) *supra*) and a corresponding control cell line expressing an inactive *CrmA* mutant. The respective MCF7 cell lines were transiently transfected with a FADD expression vector in the presence of an equimolar amount of the pCMV β-gal reporter and examined by phase contrast microscopy 24 hours later. The MCF7/mutant *CrmA* cell lines expressing FADD (β-galactosidase positive blue cells) displayed morphologic alterations typical of adherent cells undergoing apoptosis, becoming rounded, condensed, and detaching from the dish (Figure 8A, left panel). The nuclei of the rounded MCF7 cells exhibited apoptotic morphology indistinguishable from MCF7 cells treated with anti-Fas antibody in the presence of cycloheximide as assessed by propidium iodide staining. In contrast, MCF7/*CrmA* cells transfected with FADD were significantly protected from cell death and the accompanying morphologic alterations of apoptosis (Figure 8A, right panel). A quantitative representation of this data is shown in Table 1.

FADD-induced apoptosis is not peculiar to MCF7 cells, since a similar phenotype was observed in the B-cell lymphoma cell line, BJAB, which are exquisitely sensitive to anti-Fas antibody-induced apoptosis (Tewari et al. (1995) *supra*).

Delineation of the Death Effector Domain of FADD

Deletion mutants of FADD were constructed in order to determine the region responsible for initiating the cell death program (Figure 8B). As described above, MCF7 cells expressing *CrmA* and mutant *CrmA* were transiently transfected with the various FADD mutant expression vectors. Interestingly, overexpression of FADDmt,

a point mutant which fails to bind Fas, still induced cell death which was *CrmA*-inhibitable and, in fact, was slightly more potent than wild-type FADD (81% vs. 72% apoptotic cells). A FADD mutant (N-FADD) containing only 117 N-terminal amino acids was able to trigger cell death (Table 1, Figure 8B). Since a large portion of the death domain is absent from N-FADD, it is not surprising that it, like FADDmt, fails to associate with the cytoplasmic domain of Fas. N-terminal deletions of FADD (NFD-2, NFD-3, NFD-4) attenuated its ability to induce cell death (Table 1, Figure 8B). This analysis suggests that, whereas the Fas-interacting domain is in the C-terminal half of FADD, the death effector domain lies in its N-terminal portion.

Using the yeast two-hybrid screen, FADD was identified as a novel protein that associates specifically with the cytoplasmic domain of Fas (Figure 1). A BLAST search using the amino acid sequence of FADD revealed a stretch of 80 amino acids that were significantly homologous to the death domain of Fas (Figure 2B). When the region of FADD was masked, the remaining sequences did not match any proteins in the database. Interestingly, BLAST searches using the death domains of FADD, Fas and TNFR-1 revealed a significant homology to the family of ankyrins ($p<0.001$ for all three death domains). More specifically, the respective death domains aligned with approximately 80 amino acids of the negative regulatory domain of ankyrin. A previous study reported that this region of ankyrin is homologous to the cytoplasmic domain of TNFR-1 (Peters et al. (1993) Semin. in Hematol. 30:85-118), corroborating this observation. Why ankyrin contains a "death domain" remains unclear, but presumably this region is acting as a protein interaction domain.

In vitro and *in vivo* studies show that FADD specifically associates with the death domain of Fas, confirming the results of the yeast interaction assay. FADD failed to interact with Fas-LPR and Fas-FD8, a non-signaling point mutant and deletion mutant, respectively, of the Fas death domain. Interestingly, upon deletion of the negative regulatory domain of Fas, an enhanced interaction with FADD was observed. Hence, a correlation exists between the cell-killing activity of the various Fas mutants and their association with FADD (Figure 5A). A weak association between FADD and TNFR-1 was observed *in vitro* (Figure 4). In addition, β-galactosidase filter assays of yeast cotransformed with GAL4bd-Fas and GAL4ad-FADD turned blue within 1 hr, while those cotransformed with GAL4bd-TNFR-1 and

GAL4ad-FADD turned blue overnight (the other cotransformed heterologous baits remained unchanged). If the weak interaction between FADD and TNFR-1 observed in yeast and *in vitro* proves to be significant, this would correlate with the relative potencies of Fas-dependent cell death and TNF-dependent cytotoxicity (Clement, M-V. et al. (1994) J. Exp. Med. 180:557-x 567).

Having shown that FADD specifically binds the death domain of Fas, the next step was to identify the corresponding interaction domain in Fas. Previous studies have shown that death domains have a propensity to self-associate (Boldin et al. (1995) J. Biol. Chem. 270:387-391). It was thus reasonable to propose that the death domain of FADD was interacting with its homologous counterpart in Fas. As predicted, a point mutation in the death domain of FADD abrogated its association with Fas (Figure 6). These results support a model in which a death domain to death domain interaction is responsible for the binding of FADD to Fas.

Once the *in vitro* and *in vivo* association of FADD and Fas was established, the next step was to determine a functional role for this novel Fas binding protein. BJAB cells transiently transfected with AU1-FADD undergo apoptosis within 12 hours , a time frame similar to Fas-induced killing (Figures 7A and 7B). Previous studies showed that *CrmA* is a potent inhibitor of Fas-induced cell death (Tewari et al. (1995) *supra*). Likewise, *CrmA* suppressed FADD-induced cell death (Figures 7A and 7B). These functional studies, together with the biochemical data, suggests that FADD is likely a component of the Fas-signal transduction machinery. BJAB cells treated with α-Fas antibody undergo apoptosis within hours , a time frame similar to FADD-induced killing. Likewise, *CrmA*-expressing BJAB cells are resistant to both Fas- and FADD-induced killing. To ensure that the induction of cell death by FADD was not a peculiarity of one cell type, the effects of FADD on another Fas-sensitive cell line, MCF7 was also examined. As was seen in BJAB cells, overexpression of FADD in MCF7 cells induced apoptosis which was *CrmA*-inhibitable and indistinguishable from Fas-induced killing.

Deletional analysis of FADD localized the death effector domain of FADD to its N-terminal portion (Figure 8B). In fact, the N-terminal amino acids (1-117) are sufficient to cause death and deletions of the N-terminus attenuate the cell killing ability of FADD. In addition, the data refutes the possibility that FADD causes cell

death by simply aggregating the Fas antigen intracellularly, since both N-FADD and FADDmt trigger apoptosis without binding Fas.

Table 1

The Effects of Overexpressing FADD and FADD Mutants in MCF7 and BJAB cells

	Cell Lines			
	MCF7/CrmA Mutant	MCF7/CrmA	BJAB/vector	BJAB/CrmA
β-gal alone	12.9 ± 3.8	7.7 ± 4.0	6.0 ± 4.3	7.4 ± 4.3
AU1-FADD	72.1 ± 1.2	15.6 ± 4.8	91.6 ± 2.0	6.2 ± 3.6
AU1-FADDmt	81.2 ± 2.9	13.8 ± 8.4	ND	ND
AU1-N-FADD	69.9 ± 1.7	10.4 ± 6.1	ND	ND
AU1-NFD2	24.0 ± 8.8	10.9 ± 8.0	ND	ND
AU1-NFD2/6	13.6 ± 1.4	12.1 ± 7.5	ND	ND
AU1-NFD3	29.6 ± 4.2	13.0 ± 8.8	ND	ND
AU1-NFD4	21.2 ± 1.7	11.2 ± 6.2	ND	ND
AU1-TRAF1	ND	ND	5.5 ± 0.9	6.8 ± 2.6
AU1-CD40bp	ND	ND	10.8 ± 1.2	9.7 ± 1.4

Two previously characterized MCF7 and BJAB cell lines expressing *CrmA* and either a corresponding vector control line (Tewari et al., (1995) J. Biol. Chem. *supra*), or mutant *CrmA* line were transiently transfected with pCMV-β-galactosidase in the presence or absence of an approximately equimolar quantity of pcDNA3 (Invitrogen) expression constructs encoding AU1-FADD, FADD mutants (as designated in Figure 7B), AU1-TRAF1 (Rothe et al., 1994) or HA-CD40bp (Hu et al., 1994). Twenty-four hours after transection, MCF7 cells were fixed with 0.5% glutaraldehyde and stained with X-Gal for 4 hrs. The data (mean ± SEM) shown are the percentage of round blue cells among total number of blue cells counted. Round cells were confirmed to be apoptotic by propidium iodide staining of nuclei. Twelve hours after transfection, BJAB cells were cytocentrifuged, methanol fixed, and stained for β-galactosidase and with propidium iodide. The data shown are the percentage of apoptotic cells among the total number of β-galactosidase positive cells counted. The data were collected from at least 3 independent experiments. ND, not determined.

Experiment II

This experiment was conducted to show that FADD transduction is accomplished via association a class of novel and diverse signaling molecules.

Antibodies and Reagents

The anti-CD95 monoclonal antibodies used in this study include anti-Fas IgM (Upstate Biotechnology Incorporated), anti-APO-1 (IgG3, Trauth, B.C. et al. (1989) Science 245:301–305)), and PE-conjugated anti-Fas (MBL Incorporated). Anti-AU1 murine ascites was obtained from Babco, Inc. Anti-PARP antibody was clone C-2-10, which is described in Lamarre, D. et al. (1988) Biochem. Biophys. Acta. 950:147–160; it recognizes an epitope near the N-terminus of PARP located between amino acids 216 and 375. Antibodies were raised against recombinant GST-FADD fusion protein as described in Tewari et al. (1995) Cell 81:801–809. Rabbit anti-peptide antibodies (Lampire) were raised against NNKNFHKSTGMTSRSGTD of the 17 kDa subunit of Yama STAPGYYSWRNSKDGS of the 12 kDa subunit. Yama is an intracellular protein that is known in the art and described in Tewari et al. (1995) Cell, *supra*. C2-ceramide (D-erythro) and C2-dihydroceramide (D-erythro) were purchased from Matreya, Inc. and dissolved in ethanol.

Cell Lines and Culture

The B lymphoma cell line BJAB and the breast carcinoma cell line MCF7 were grown in RPMI 1640 complete medium (10% heat-inactivated fetal bovine serum (Hyclone), L-glutamine, penicillin/streptomycin, and non-essential amino acids). MCF7-Fas cells, as described above, were grown in RPMI 1640 complete media supplemented with 0.5 mg/ml G418 (Gibco BRL). BJAB and MCF7 stable cell lines were grown in complete media supplemented with 3 mg/ml and 0.5 mg/ml G418, respectively.

Stable and Transient Transfections

To generate the pooled stable cell lines, BJAB-FADD-DN and MCF7-FADD-DN, cells were transfected with pcDNA3-FADD-DN as described above.

From the pooled populations, individual clones were obtained and plated in duplicate on 48-well Costar plates. One set of cells was treated with anti-Fas IgM (100 ng/ml) and clones resistant to Fas-induced cell death were identified. The untreated, resistant clones were then pooled to obtain BJAB-sFADD-DN (which represents a pool of 7 resistant clones) and MCF7-sFADD-DN (which represents a pool of 9 resistant clones). All stable lines generated were assessed for expression of FADD-DN by immunoblotting. MCF7 cells were transiently transfected with lipofectamine as described above.

Cell Culture, Cell Death and Viability Assays

To assess nuclear morphology, fluorescent DNA-staining dyes were utilized as described in Tewari et al. (1995) J. Biol. Chem. *supra.* DNA fragmentation (TUNEL staining) was determined using the In Situ Cell Death Detection Kit (Boehringer Mannheim). BJAB cells were air-dried onto Colorfrost/Plus microscope slides (Fisher Scientific) using 4% paraformaldehyde and the manufacturer's protocol followed. The TUNEL-stained cells were then counterstained with propidium iodide (10 μg/ml) and visualized by fluorescence microscopy.

To assess cell viability, the MTT Conversion Assay was utilized as follows and as described in Opipari, A.W. et al. J. Biol. Chem. (1992) 267:12424-12427 and crystal violet staining as described in Tartaglia, L.A. et al. (1993) Cell 74:845-853.

Cell Cultures-MCF7 cells, BJAB cells, and derived vector and *CrmA* stable transfectants along with the *CrmA* mutant-transfected stable lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), L-glutamine, penicillin/streptomycin, and nonessential amino acids and additionally supplemented with G418 sulfate (Life Technologies, Inc.) to 500 μg/ml for MCF7 transfectants and 3 mg/ml for BJAB transfectants.

Ceramide Assays

Ceramide levels were determined by a modified diacylglycerol kinase (DGK) as described in Tepper C. (1995), PNAS 92:8443–8447; Preiss, J. (1986) J. Biol. Chem. 261:8597–8600; and Jayadev, S. (1994) J. Biol. Chem. 269:5757–5763.

Immunoprecipitati ns, Western Blotting and 2D Gels

Immunoblotting of cell lysates for PARP was carried out as described in Tewari (1995) Cell *supra*. Yama processing was assessed using 1 X 10^7 BJAB-vector and BJAB-sFADD-DN cells untreated or treated with 100ng/ml antiFas IgM for 18 hrs. Cells were then lysed in 60µl of 0.1% NP40, free-thawed 3X, and centrifuged at 14,000 rpm for 20 minutes. Cytoplasmic extracts were carefully added to sample buffer and run on a 15% gel, transferred to a nitrocellulose membrane and immunoblotted with antibodies directed against the 17 kDa and 12 kDa subunits of Yama. To show expression of FADD-DN in BJAB and MCF7 cells, cells were immunoprecipitated in PBS-TDS with anti-AU1 antibody (1:100) and Western blotted with anti-FADD polyclonal antisera (1:1000).

Immunoprecipitation of the DISC and analysis on 2D gels was done as previously described Kischkel, F.C. (1995) EMBO 14:5579–5588. Alternatively, cell lysates were immunoprecipitated with anti-AU1 antibody coupled to Protein A Sepharose beads as described in Kischkel, F.C. (1995) *supra*.

NF-κB assay

MCF7-vector and MCF7-sFADD-DN cells were transfected with the NF-κB-dependent E-selectin reporter construct and luciferase activity assessed as described in Rothe (1995) Science 269:1424–1427.

Overexpression of FADD causes apoptosis, resulting in cleavage of the death substrate poly (ADP-ribose) polymerase (PARP) to signature apoptotic fragments. The deletion mutant, NFD4, was able to interact with CD95, but failed to initiate apoptosis, showing that it may have a dominant negative effect on CD95 signaling. FADD-DN lacks 80 N-terminal amino acids but contains the death domain responsible for association with the related death domain of CD95. The B lymphoma cell line BJAB was transfected with either the expression vector pcDNA3 alone or as a FADD-DN expression construct. Stable transfectants were generated by neomycin (G418) selection and pooled populations assessed for FADD-DN expression and sensitivity to anti-Fas-induced apoptosis (BJAB-FADD-DN). Expression of FADD-DN in both a pooled population and in a mixture of selected clones (BJAB-sFADD-

DN) dramatically abrogated CD95-induced cell death. The apoptotic nature of the cell death was confirmed by the TUNEL assay which detects 3'-OH DNA strand breaks. The FADD-DN expressing BJAB cells were not inherently resistant to apoptotic cell death since the protein kinase inhibitor staurosporine and the calcium ionophore A23187 equally killed the three cell lines. CD95 surface expression was equivalent in the vector and FADD-DN cell lines as assessed by flow cytometry.

To determine whether the ceramide signaling event was blocked by the FADD derivative, BJAB cells expressing FADD-DN were treated with anti-Fas IgM and, subsequently, ceramide levels assessed. Consistent with a proximal role of FADD in CD95 signaling, FADD-DN inhibited CD95-mediated ceramide generation (Figure 9B). Additionally, vector and FADD-DN transfected BJAB cells were equally susceptible to cell death induced by the cell-permeable, active ceramide analogue, C_2-ceramide, confirming that the block in the death pathway was prior to ceramide generation (Figure 9C).

Mammalian homologs of the *Caenorhabditis elegans* cell death protease CED-3 are thought to be distal effectors of the CD95 cell death pathway. Yama is an intracellular protein known in the art and described in Tewari et al. (1995) Cell, *supra* which is activated by CD95 engagement (see Figure 9C). Endogenous Yama is expressed as a 32 kD pro-enzyme and upon activation is proteolytically processed into active 17 kDa and 12 kDa subunits. One of the proposed substrates of Yama is the nuclear enzyme PARP as described in Tewari et al. (1995) Cell *supra*. As expected, CD95-mediated activation of Yama and resulting PARP cleavage was blocked by FADD-DN (Figure 9D).

In the yeast two-hybrid assay, FADD had a weak but specific interaction with TNFR-1. To determine whether FADD has a role in TNFR-1 induced cell death, the FADD derivative was transfected into TNF-sensitive MCF7 breast carcinoma cells and stable cell lines generated. Interestingly, FADD-DN expressing MCF7 cells were equally resistant to both CD95- and TNF-induced cell death (Figure 10A, Table 2), suggesting a proximal convergence of the cytokine-mediated cell death pathway. Additionally 293T cells transiently overexpressing TNFR-1 were protected from cell death by co-transfecting FADD-DN. As with the BJAB cell lines, the FADD-DN MCF7 cell lines were not resistant to staurosporine-induced apoptosis (Table 2).

Overexpression of interleukin-1β converting enzyme (ICE) could "bypass" the dominant negative effect of the FADD derivative, suggesting that the death pathway was being blocked upstream of the ICE-like proteases implicated in the apoptotic pathway.

While the main activity of CD95 is to trigger apoptosis, TNFR-1 can signal an array of diverse pro-inflammatory and immunoregulatory activities. Distinct from CD95, TNFR-1 is an inducer of nuclear factor κB (NF-κB). MCF7-vector and MCF7-sFADD-DN cells were transfected with an NF-κB dependent reporter gene and relative NF-κB activity assessed (Figure 10B). In both cell lines, NF-κB was activated equally well, suggesting that TNFR-1 utilizes FADD to transduce the death signal and activates NF-κB by a different mechanism.

To determine the mechanism by which the FADD derivative exerts its dominant negative effect, co-immunoprecipitation of FADD and FADD-DN with CD95 or TNFR-1 was assessed. 293T cells were co-transfected with AU1-epitope tagged FADD constructs and FLAG-epitope tagged CD95, FLAG-TNFR-1, or FLAG-B94. Cell lysates were immunoprecipitated with anti-FADD antibody and subsequently immunoblotted with anti-FLAG antibody. TNFR-1 and CD95, but not a control cytoplasmic protein, B94, co-immunoprecipitated with FADD-DN (Figure 11A). The association of FADD and FADD-DN with CD95 was ten-fold greater than with TNFR-1, correlating with the relative apoptotic potential of the two death receptors. Thus, FADD-DN exerts its inhibitory action by directly or indirectly forming a complex with the death receptors, preventing recruitment of endogenous FADD.

In the case of CD95, the endogenous signaling machinery was studied. Four proteins, termed CAPs (for Cytotoxicity-dependent APO-1 associated proteins), associate with CD95 in a ligand-dependent fashion. The oligomerized receptor, along with the associated CAPs, has been designated the CD95 death inducing signaling complex (DISC). CAP3 or CAP4 are not the recently described candidate signaling molecules FAP-1 or RIP, as anti-peptide antibodies capable of detecting endogenous FAP-1 or RIP, respectively, were unable to detect either protein associated with activated or unactivated CD95. As expected, in vector transfected BJAB cells, the DISC formed upon anti-APO-1 treatment (Figure 11B). By contrast, anti-APO-1

stimulated sFADD-DN cells did not form a complete DISC. Instead, FADD-DN complexed with CD95 in a ligand-dependent fashion and inhibited the recruitment of FADD, CAP3 and CAP4, thereby disrupting the DISC (Figure 11B). Similar results were obtained using FADD-DN expressing MCF7 cells. Thus, the N-terminus of FADD is required for the recruitment and assembly of the downstream DISC components, CAP3 and CAP4.

Thus, FADD is established as the proximal signal transduction of CD95. Many of the contenders, including RIP and FAP1, were not found associated with the active or inactive receptor, nor are dominant negative inhibitors likely to exist. Of paramount importance is the fact that the dominant negative version of FADD potently abrogates TNF-induced apoptosis but not TNF-induced NF-κB activation. This FADD likely is the common conduit of the cytokine-mediated death signal and also demonstrates that the signaling pathways for TNF-induced apoptosis and NF-κB activation are distinct (Figure 11C). FADD is both a necessary and sufficient mediator of CD95 and TNFR-1-induced apoptosis as overexpression of FADD engages the cell death machinery, while a truncated derivative acts as a potent dominant negative regulator.

TABLE 2

Expression of FADD-DN in MCF7 cells blocks CD95- and TNF-induced apoptosis

MCF7 stable cell lines were generated using the method described for the BJAB stable lines. MCF7-FADD-DN is a pooled population of transfectants while the MCF7-sFADD-DN is a mixture of selected clones. Expression was confirmed by Western blotting for FADD-DN (Figure 10A). The indicated MCF7 cell lines were transiently transfected with pCMV-β-gal (0.25 μg), pcDNA3-Fas (0.1 μg) and control vector DNA (1.0 μg) or pcDNA3-AU1-hICE (1.0 μg). Twenty-four hrs. after transfection, cells were treated with anti-Fas IgM (250 ng/ml), TNF (100 ng/ml) or 1mM staurosporine (Sigma). Transfection and X-gal staining were done as previously described in Boldin, M.D. et al. (1995) J. Biol. Chem. 270:7795-7798. The data (mean ± SD; n of at least 300 cells) represents the percentage of non-apoptotic cells. Round cells were confirmed to be apoptotic by propidium iodide staining of nuclei.

% Non-Apoptotic Cells			
	MCF7-Vector	MCF7-FADD-DN	MCF7-sFADD-DN
UnRx	85.8 ± 5.0	82.9 ± 5.5	86.4 ± 0.8
anti-Fas Igm	18.1 ± 6.0	51.0 ± 5.7	80.5 ± 3.7
TNF	16.7 ± 7.6	52.5 ± 4.5	83.9 ± 1.6
staurosporine	3.6 ± 1.8	2.8 ± 2.1	5.1 ± 3.0
ICE	35.5 ± 1.8	26.5 ± 8.7	26.2 ± 4.4

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and the examples are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

What is claimed is:

1. A protein characterized by having the ability to bind the cytoplasmic region of a Fas receptor.

2. Purified mammalian protein of claim 1.

3. A polypeptide fragment of the protein of claim 1.

4. A FADD protein or polypeptide that has been recombinantly produced and isolated from a host cell.

5. A nucleic acid molecule coding for a protein or polypeptide characterized by having the ability to bind the cytoplasmic domain of the Fas receptor.

6. An antibody capable of specifically forming an antibody complex with the protein of claims 1 or 2.

7. An agent characterized by having the ability to inhibit the binding of the mammalian protein of claim 1 to the cytoplasmic domain of a Fas receptor.

8. An agent characterized by inhibiting Fas-associated apoptotic cell death.

9. A hybridoma cell line which produces the antibody of claim 15.

10. A method of modulating a cellular function regulated by the FADD pathway in a suitable cell, which comprises introducing into the cell a FADD nucleic acid and growing the cell under suitable conditions such that the nucleic acid is transcribed and translated into FADD protein in the cell.

11. A method for screening for an agent useful to modulate cellular function regulated by the FADD pathway, the method comprising the steps of:

a) providing a FADD domain receptor known to bind FADD to a solid support;

b) contacting the agent to be tested with the receptor bound support of step a) under conditions favoring binding of the domain to FADD;

c) contacting detectably-labeled FADD to the solid support of step b) under conditions favoring binding of the domain receptor to FADD;

d) detecting the presence of any complex formed between the domain receptor and FADD to form receptor-FADD complex;

e) the absence of complex being indicative that the agent inhibits binding of FADD to the receptor; and

f) analyzing the results of step d) to determine how the agent modulates the cellular function regulated by the FADD pathway.

12. A method for screening for an agent useful to modulate cellular function regulated by the FADD receptor, the method comprising the steps of:

a) providing a FADD domain receptor known to bind FADD bound to a solid support;

b) contacting detectably-labeled FADD to the solid support of step a) under conditions favoring binding of the domain receptor to FADD;

c) contacting the agent to be screened with the receptor bound support of step b) under conditions favoring binding of the domain to the receptor to FADD;

d) detecting the presence of any complex formed between domain receptor and FADD to form receptor-FADD complex; and

e) the absence of complex being indicative that the agent competitively inhibits binding of FADD to the receptor; and

f) analyzing the results of step e) to determine how the agent modulates the cellular function regulated by the FADD receptor pathway.

13. The agents identified by the methods of claims 11 or 12.

14. The method of claim 11 or 12, wherein the FADD domain receptor is Fas, TNFR-1, or CAPP3/CAPP4.

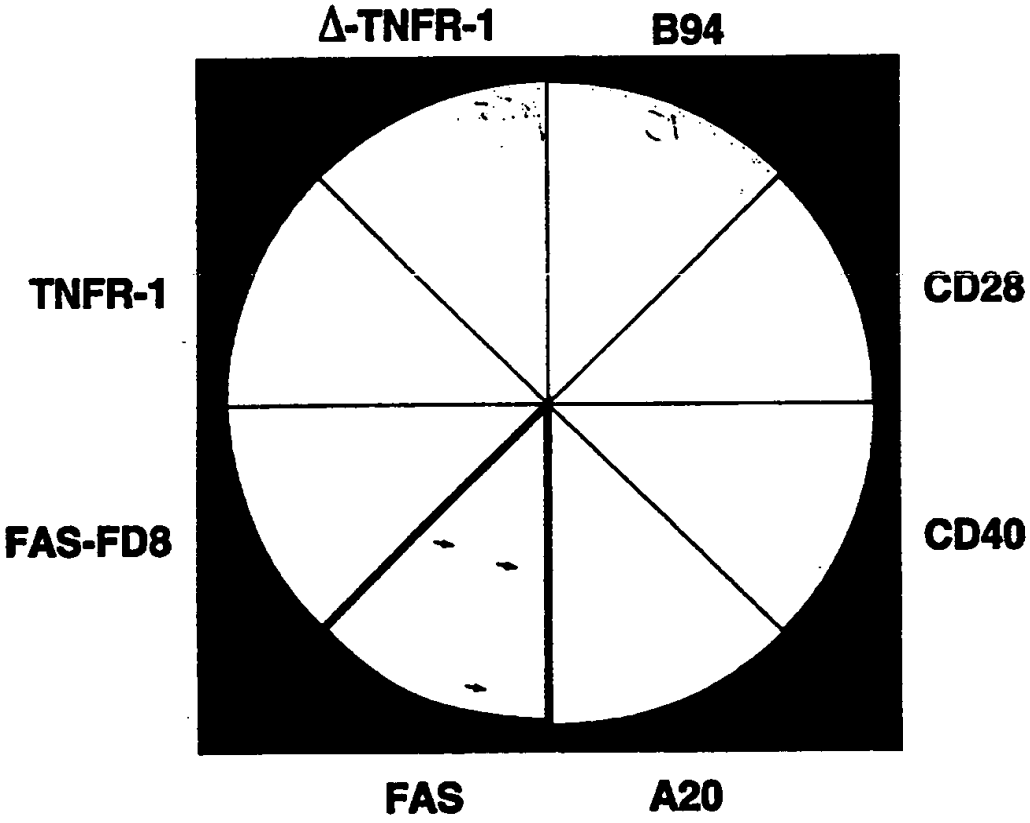

FIG. 1

CTC TAA AGGT TCGGGGGTGG AATCCTTGGG CCGCTGGGCA AGCGGCGAGA CCTGGCCAGG

100

GCCAGCGAGC CGAGGACAGA GGGCGCACGG AGGGCCGGGC CGCAGCCCCG GCCGCTTGCA

GACCCCGCC ATG GAC CCG TTC CTG GTG CTG CTG CAC TCG GTG TCG TCC
Met Asp Pro Phe Leu Val Leu Leu His Ser Val Ser Ser

200 CLONE 15

AGC CTG TCG AGC AGC GAG CTG ACC GAG CTC AAG TTC CTA TGC CTC GGG
Ser Leu Ser Ser Ser Glu Leu Thr Glu Leu Lys Phe Leu Cys Leu Gly

CLONE 8

CGC GTG GGC AAG CGC AAG CTG GAG CGC GTG CAG AGC GGC CTA GAC CTC
Arg Val Gly Lys Arg Lys Leu Glu Arg Val Gln Ser Gly Leu Asp Leu

300

TTC TCC ATG CTG CTG GAG CAG AAC GAC CTG GAG CCC GGG CAC ACC GAG
Phe Ser Met Leu Leu Glu Gln Asn Asp Leu Glu Pro Gly His Thr Glu

CTC CTG CGC GAG CTG CTC GCC TCC CTG CGG CGC CAC GAC CTG CTG CGG
Leu Leu Arg Glu Leu Leu Ala Ser Leu Arg Arg His Asp Leu Leu Arg

400

CGC GTC GAC GAC TTC GAG GCG GGG GCG GCG GCC GGG GCC GCG CCT GGG
Arg Val Asp Asp Phe Glu Ala Gly Ala Ala Ala Gly Ala Ala Pro Gly

GAA GAA GAC CTG TGT GCA GCA TTT AAC GTC ATA TGT GAT AAT GTG GGG
Glu Glu Asp Leu Cys Ala Ala Phe Asn Val Ile Cys Asp Asn Val Gly

▼ 500

AAA GAT TGG AGA AGG CTG GCT CGT CAG CTC AAA GTC TCA GAC ACC AAG
Lys Asp Trp Arg Arg Leu Ala Arg Gln Leu Lys Val Ser Asp Thr Lys

ATC GAC AGC ATC GAG GAC AGA TAC CCC CGC AAC CTG ACA GAG CGT GTG
Ile Asp Ser Ile Glu Asp Arg Tyr Pro Arg Asn Leu Thr Glu Arg Val

600

CGG GAG TCA CTG AGA ATC TGG AAG AAC ACA GAG AAG GAG AAC GCA ACA
Arg Glu Ser Leu Arg Ile Trp Lys Asn Thr Glu Lys Glu Asn Ala Thr

GTG GCC CAC CTG GTG GGG GCT CTC AGG TCC TGC CAG ATG AAC CTG GTG
Val Ala His Leu Val Gly Ala Leu Arg Ser Cys Gln Met Asn Leu Val

GCT GAC CTG GTA CAA GAG GTT CAG CAG GCC CGT GAC CTC CAG AAC AGG
Ala Asp Leu Val Gln Glu Val Gln Gln Ala Arg Asp Leu Gln Asn Arg

FIG. 2A

700
AGT GGG GCC ATG TCC CCG ATG TCA TGG AAC TCA GAC GCA TCT ACC TCC
Ser Gly Ala Met Ser Pro Met Ser Trp Asn Ser Asp Ala Ser Thr Ser

GAA GCG TCC TGA TGGGCCGCTG CTTTGCGCTG GTGGACCACA GGCATCTACA
Glu Ala Ser *

800
CAGCCTGGAC TTTGGTTCTC TCCAGGAAGG TAGCCCAGCA CTGTGAAGAC CCAGCAGGAA

900
GCCAGGCTGA GTGAGCCACA GACCACCTGC TTCTGAACTC AAGCTGCGTT TATTAATGCC

TCTCCCGCAC CAGGCCGGGC TTGGGCCCTG CACAGATATT TCCATTTCTT CCTCACTATG

1000
ACACTGAGCA AGATCTTGTC TCCACTAAAT GAGCTCCTGC GGGAGTAGTT GGAAAGTTGG

AACCGTGTCC AGCACAGAAG GAATCTGTGC AGATGAGCAG TCACACTGTT ACTCCACAGC

1100
GGAGGAGACC AGCTCAGAGG CCCAGGAATC GGAGCGAAGC AGAGAGGTGG AGAACTGGGA

1200
TTTGAACCCC CGCCATCCTT CACCAGAGCC CATGCTCAAC CACTGTGGCG TTCTGCTGCC

CCTGCAGTTG GCAGAAAGGA TGTTTTGTCC CATTTCCTTG GAGGCCACCG GGACAGACCT

1300
GGACACTAGG GTCAGGCGGG GTGCTGTGGT GGGGAGAGGC ATGGCTGGGG TGGGGGTGGG

GAGACCTGGT TGGCCGTGGT CCAGCTCTTG GCCCCTGTGT GAGTTGAGTC TCCTCTCTGA

1400
GACTGCTAAG TAGGGGCAGT GATGGTTGCC AGGACGAATT GAGATAATAT CTGTGAGGTG

1500
CTGATGAGTG ATTGACACAC AGCACTCTCT AAATCTTCCT TGTGAGGATT ATGGGTCCTG

CAATTCTACA GTTTCTTACT GTTTTGTATC AAAATCACTA TCTTTCTGAT AACAGAATTG

1600
CCAAGGCAGC GGGATCTCGT ATCTTTAAAA AGCAGTCCTC TTATTCCTAA GGTAATCCTA
TTAAAA

FIG. 2B

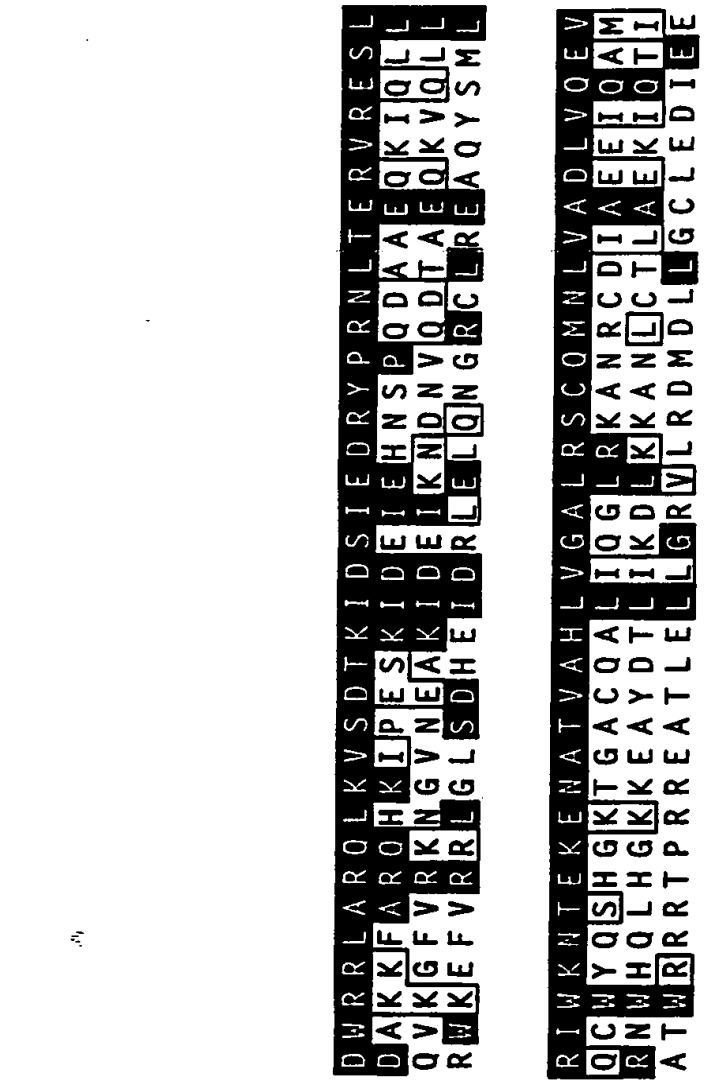
hFADD (111-145) DWRRLARQLKVSDTKIDSIEDRYPRNLTERVRESL
rFas (217-251) DAKKFARQHKIPESKIDEIEHNSPQDAAEQKIQLL
hFas (228-262) QVKGFVRKNGVNEAKIDEIKNDNVQDTAEQKVQLL
hTNFR-1 (341-375) RWKEFVRRLGLSDHEIDRLELQNGRCLREAQYSML
hFADD (146-180) RIWKNTEKENATVAHLVGALRSCQMNLVADLVQEV
rFas (252-286) QCWYQSHGKTGACQALIQGLRKANRCDIAEEIQAM
hFas (263-297) RNWHQLHGKKEAYDTLIKDLKKANLCTLAEKIQTI
hTNFR-1 (376-410) ATWRRTPRREATLELLGRVLRDMDLLGCLEDIEE

FIG. 2C

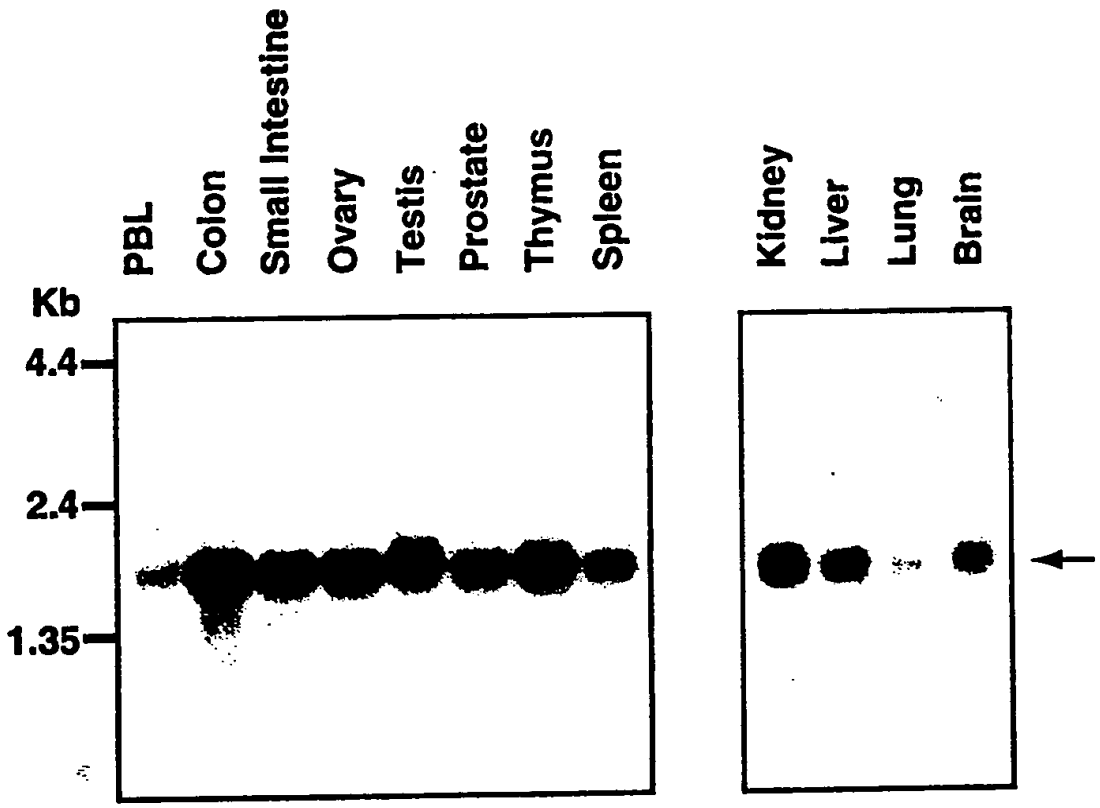
PBL
Colon
Small Intestine
Ovary
Testis
Prostate
Thymus
Spleen
Kidney
Liver
Lung
Brain
Kb
4.4
2.4
1.35

FIG. 3A

FIG. 3B

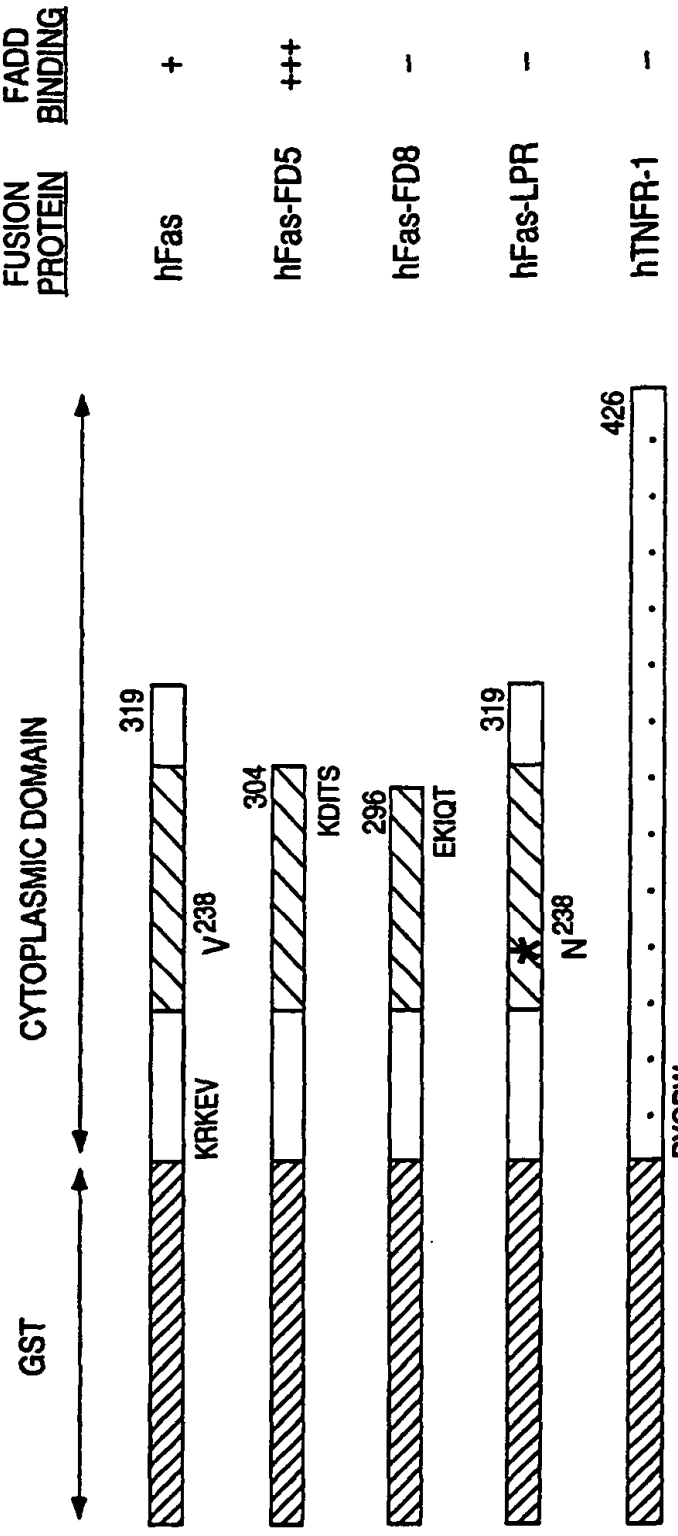
FUSION PROTEIN
FADD BINDING
hFas +
hFas-FD5 +++
hFas-FD8 -
hFas-LPR -
hTNFR-1 -
CYTOPLASMIC DOMAIN
GST
319
KRKEV
V238
304
KDITS
296
EKIQT
319
N238
426
RYQRW

FIG. 4A

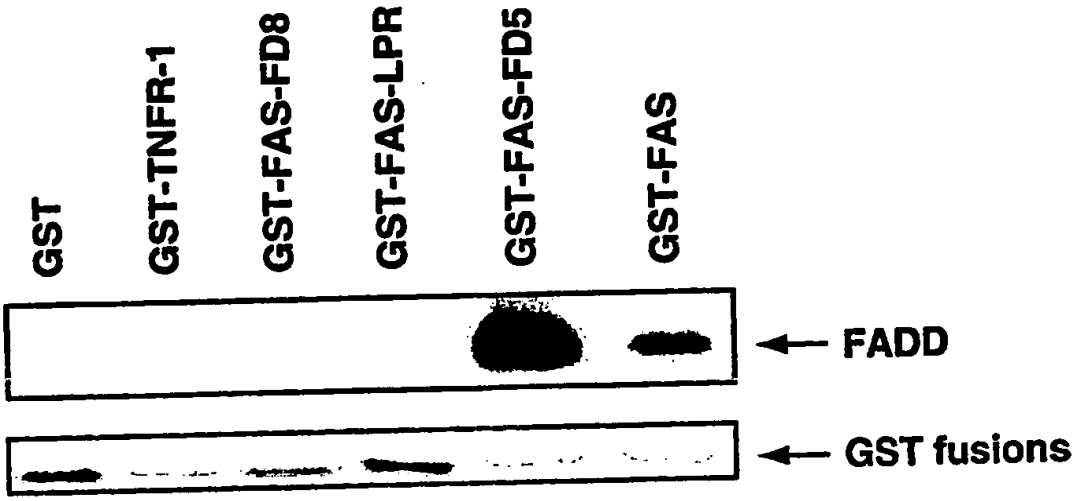
GST
GST-TNFR-1
GST-FAS-FD8
GST-FAS-LPR
GST-FAS-FD5
GST-FAS
FADD
GST fusions

FIG. 4B

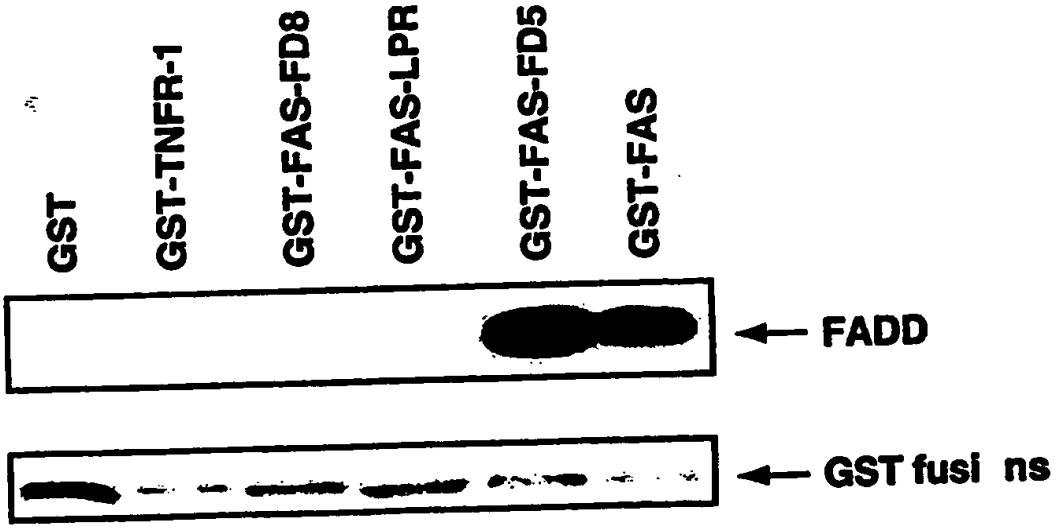
GST
GST-TNFR-1
GST-FAS-FD8
GST-FAS-LPR
GST-FAS-FD5
GST-FAS
FADD
GST fusi ns

FIG. 4C

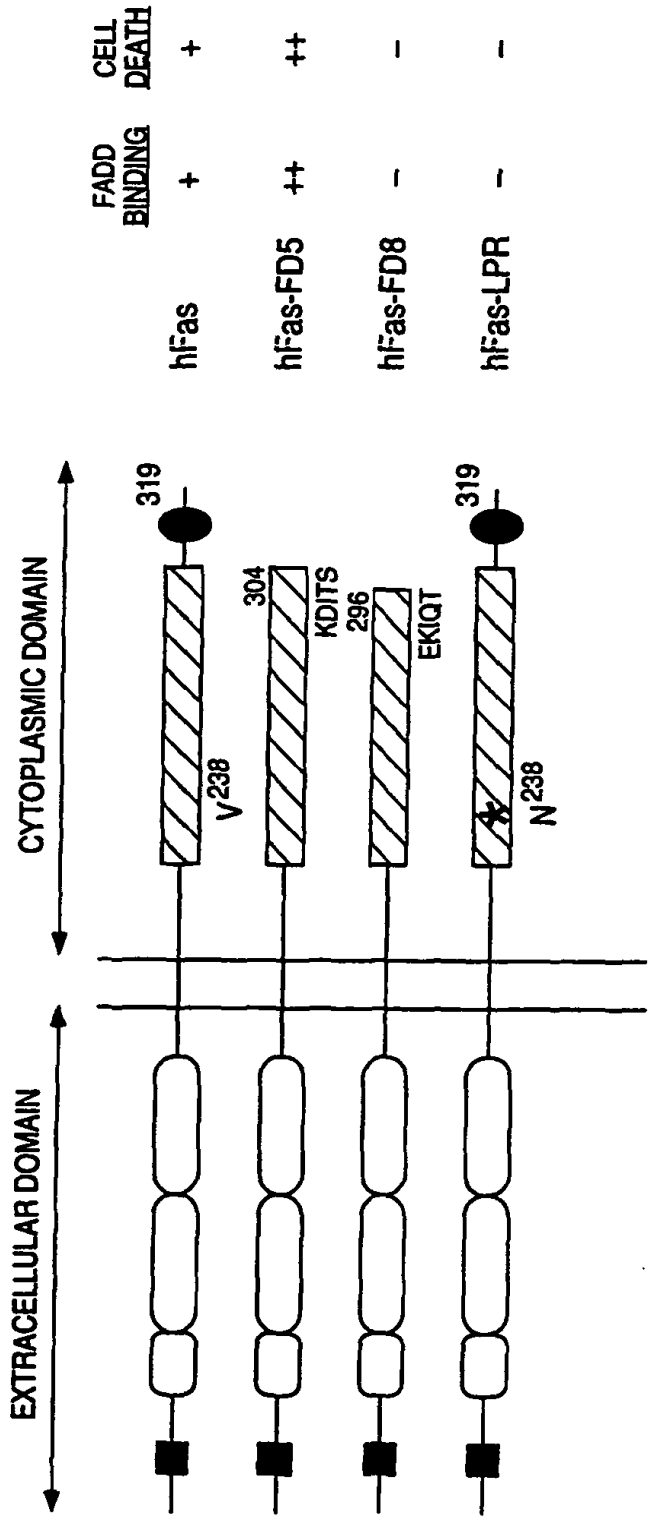
CELL DEATH
FADD BINDING
hFas + +
hFas-FD5 ++ ++
hFas-FD8 - -
hFas-LPR - -
319
319
304
KDITS
296
EKIQT
V238
N238
CYTOPLASMIC DOMAIN
EXTRACELLULAR DOMAIN

FIG. 5A

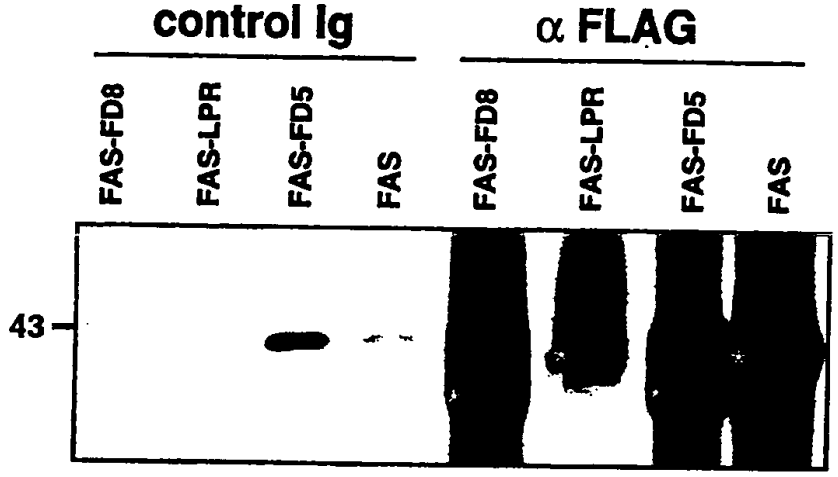
control Ig
α FLAG
FAS-FD8
FAS-LPR
FAS-FD5
FAS
FAS-FD8
FAS-LPR
FAS-FD5
FAS
43

FIG. 5B

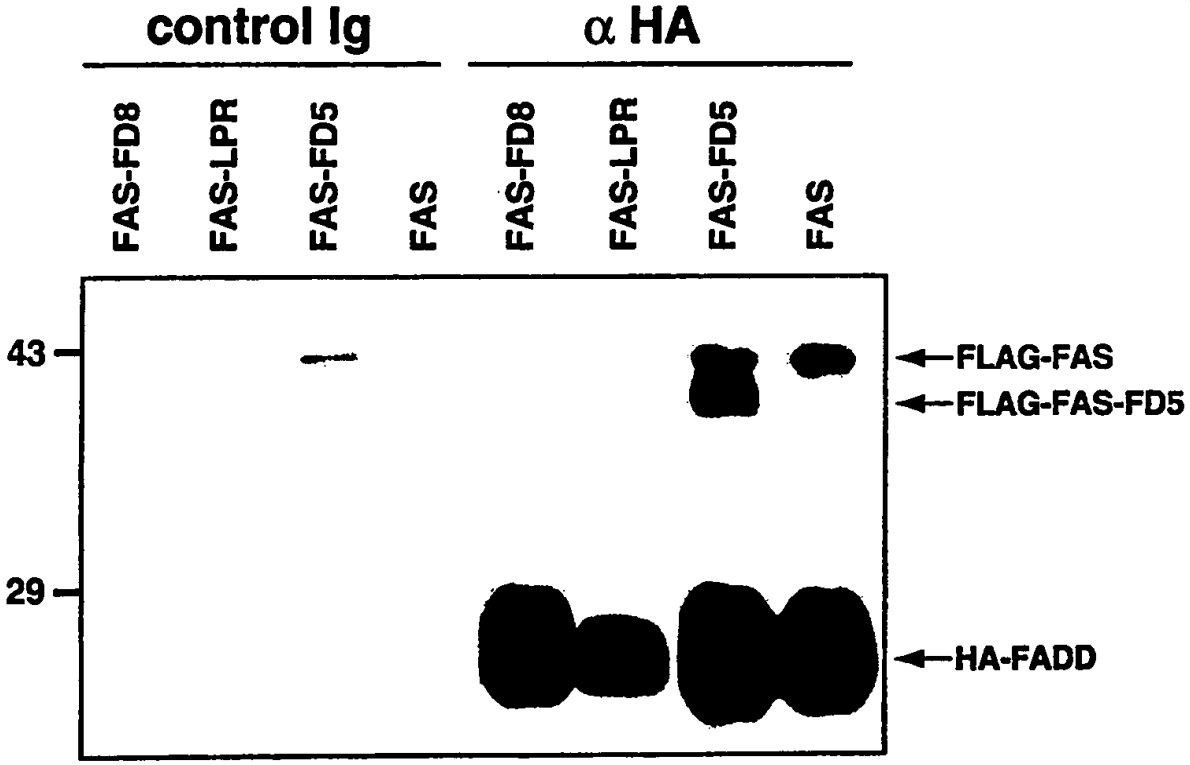
control Ig
α HA
FAS-FD8
FAS-LPR
FAS-FD5
FAS
FAS-FD8
FAS-LPR
FAS-FD5
FAS
43
29
FLAG-FAS
FLAG-FAS-FD5
HA-FADD

FIG. 5C

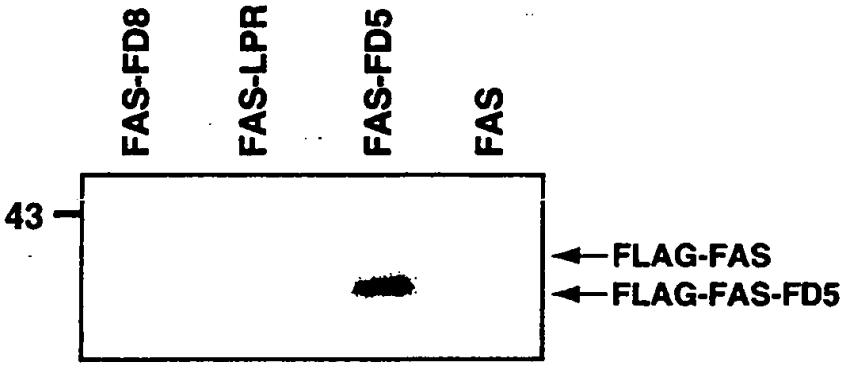

FIG. 5D

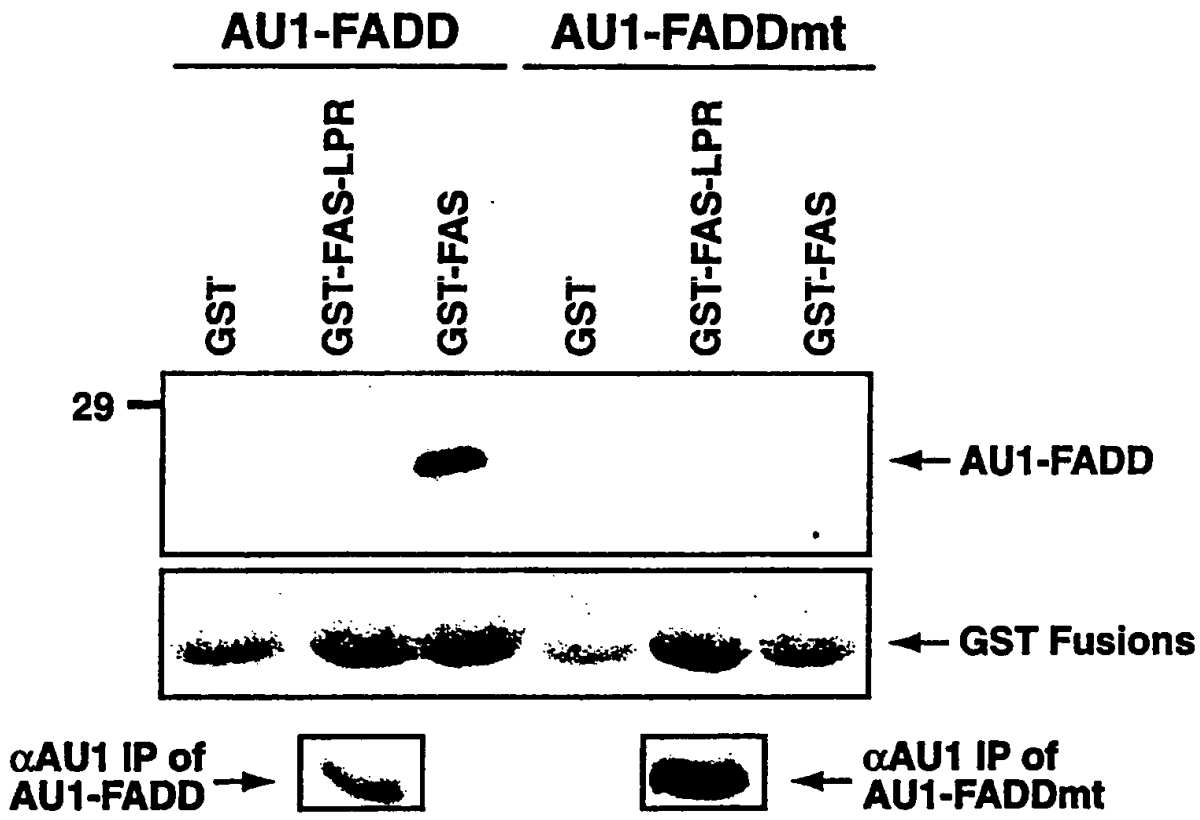
AU1-FADD
AU1-FADDmt
GST
GST-FAS-LPR
GST-FAS
GST
GST-FAS-LPR
GST-FAS
29
AU1-FADD
GST Fusions
αAU1 IP of AU1-FADD
αAU1 IP of AU1-FADDmt

FIG. 6

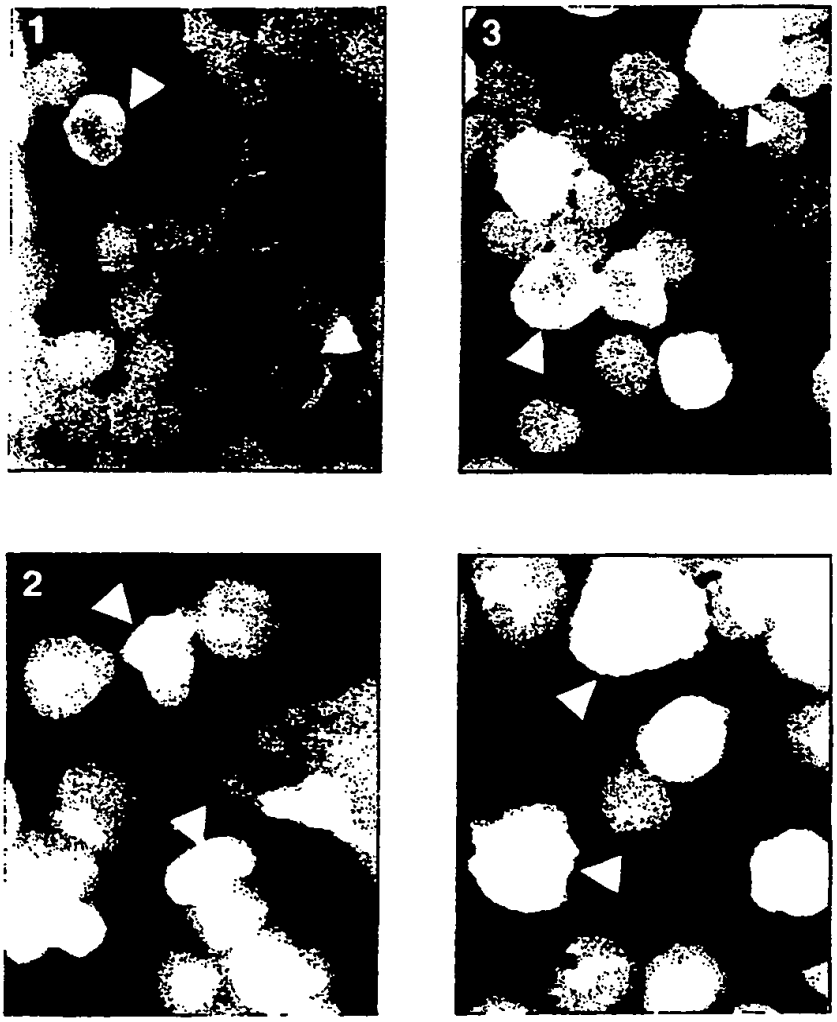

FIG. 7A

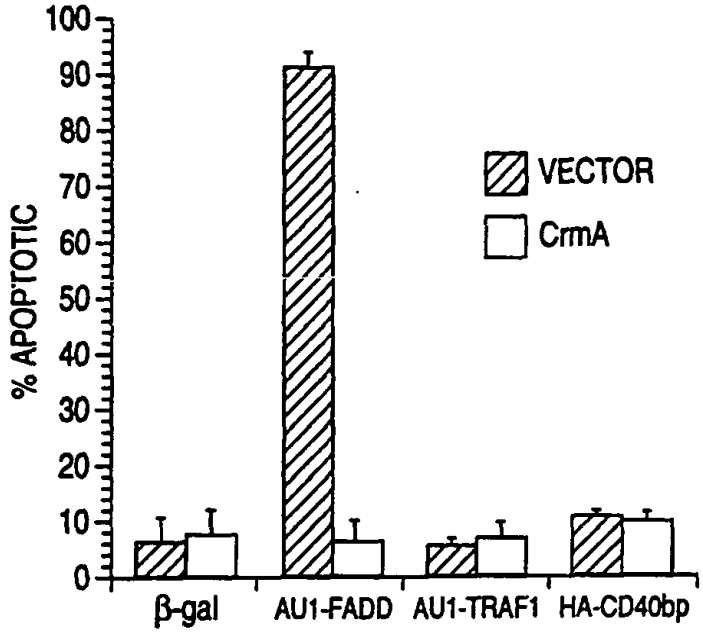
100
90
80
70
60
50
40
30
20
10
0
% APOPTOTIC
VECTOR
CrmA
β-gal
AU1-FADD
AU1-TRAF1
HA-CD40bp

FIG. 7B

FIG. 7C

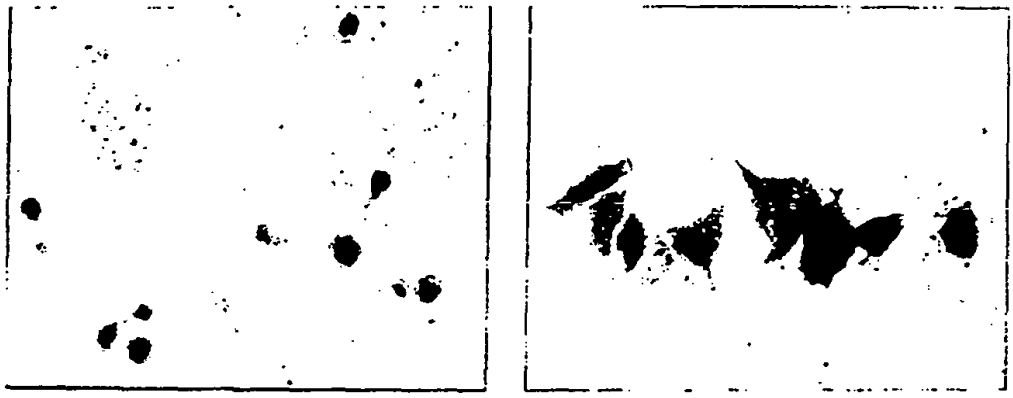

FIG. 8A

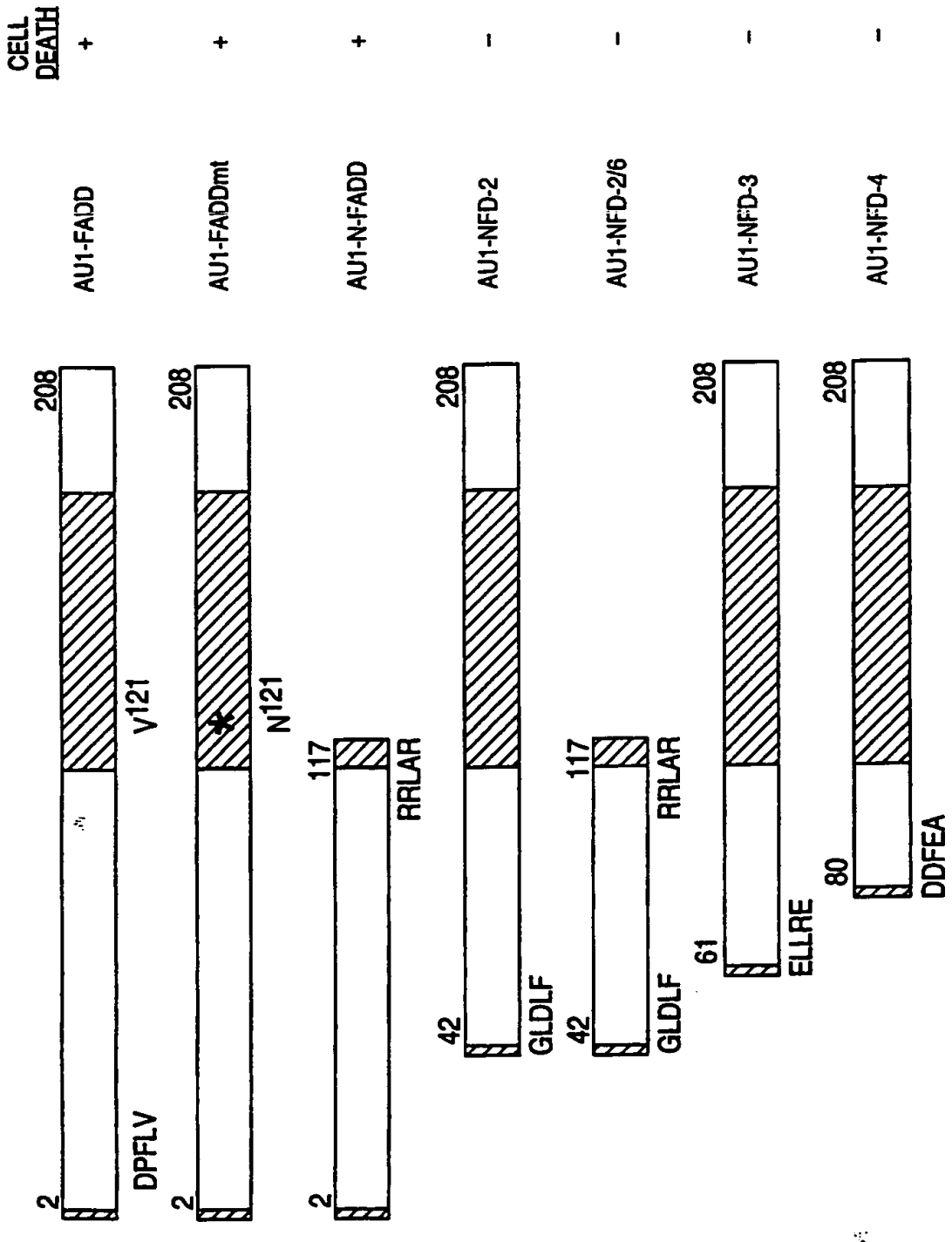
CELL DEATH
AU1-FADD
+
AU1-FADDmt
+
AU1-N-FADD
+
AU1-NFD-2
-
AU1-NFD-2/6
-
AU1-NFD-3
-
AU1-NFD-4
-
208
2
DPFLV
V121
208
2
N121
117
2
RRLAR
208
42
GLDLF
117
42
GLDLF
RRLAR
208
61
ELLRE
208
80
DDFEA

FIG. 8B

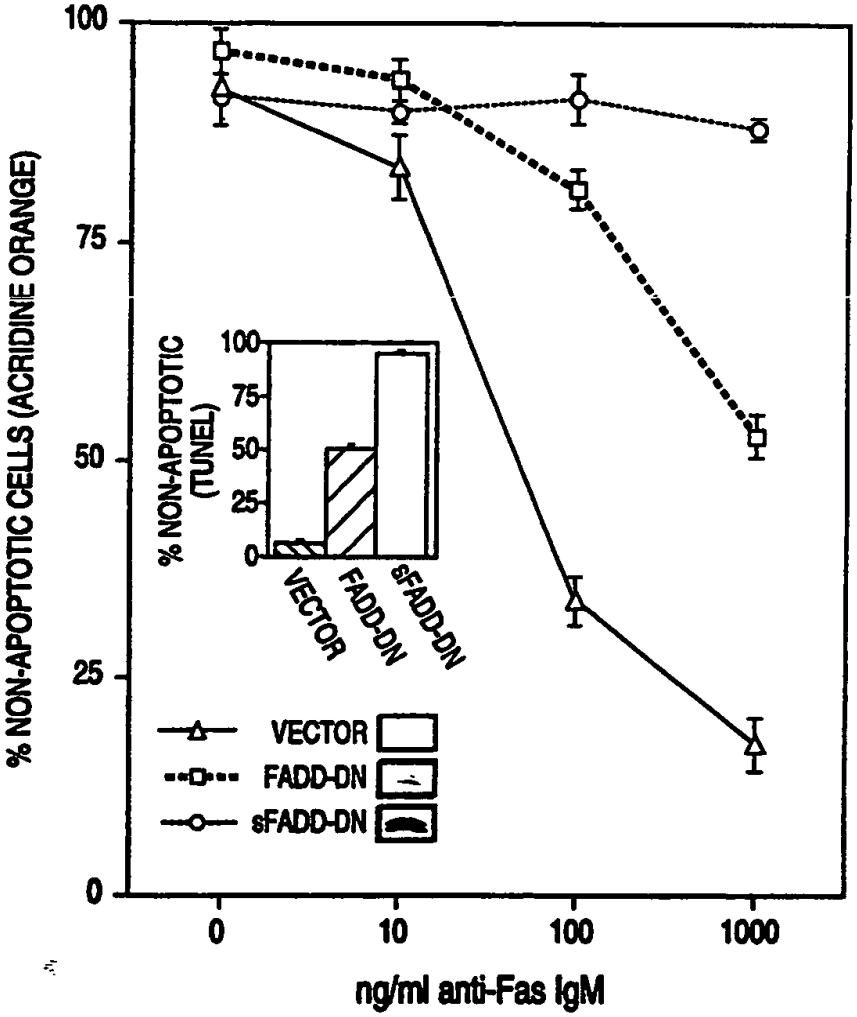
100
75
50
25
0
% NON-APOPTOTIC CELLS (ACRIDINE ORANGE)
0
10
100
1000
ng/ml anti-Fas IgM
VECTOR
FADD-DN
sFADD-DN
% NON-APOPTOTIC (TUNEL)
100
75
50
25
0
VECTOR
FADD-DN
sFADD-DN

FIG. 9A

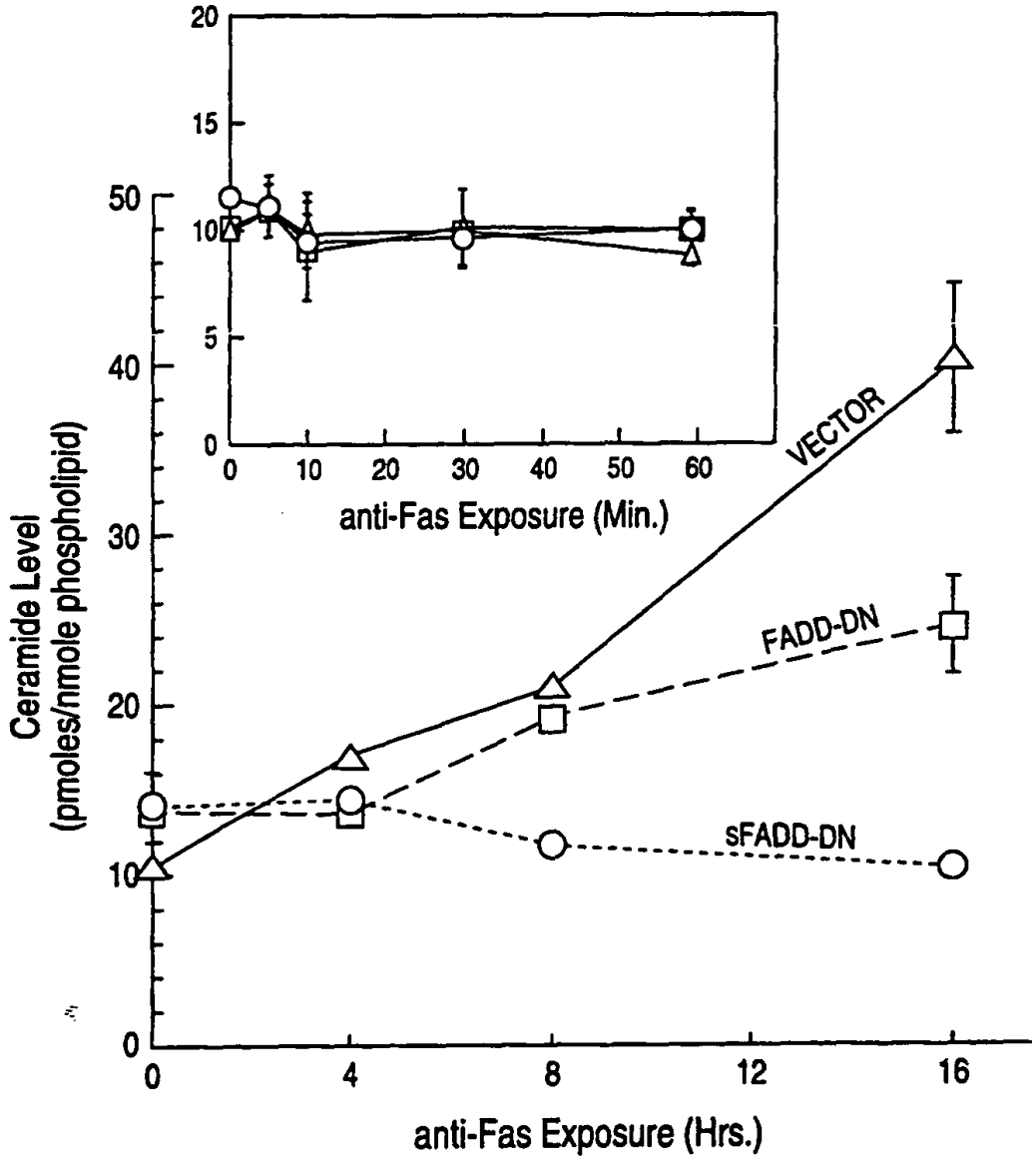
Ceramide Level
(pmoles/nmole phospholipid)
anti-Fas Exposure (Hrs.)
anti-Fas Exposure (Min.)
VECTOR
FADD-DN
sFADD-DN
0
4
8
12
16
10
20
30
40
50
0
5
10
15
20
0
10
20
30
40
50
60

FIG. 9B

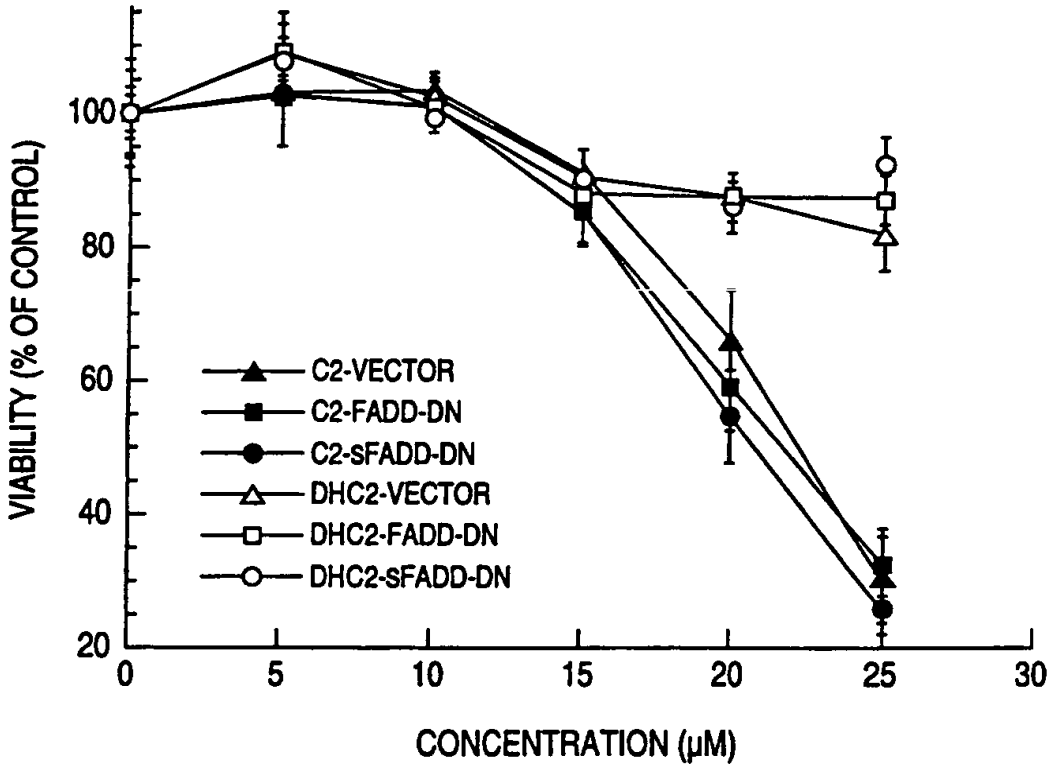
VIABILITY (% OF CONTROL)
100
80
60
40
20
0
5
10
15
20
25
30
CONCENTRATION (μM)
C2-VECTOR
C2-FADD-DN
C2-sFADD-DN
DHC2-VECTOR
DHC2-FADD-DN
DHC2-sFADD-DN

FIG. 9C

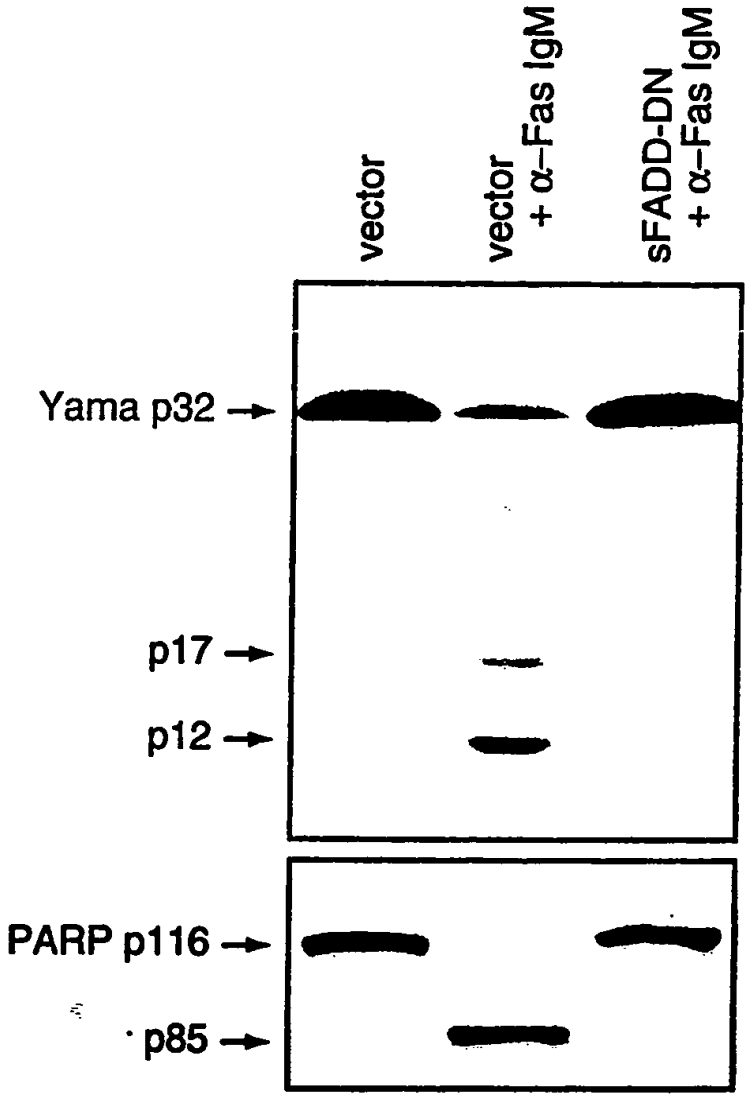
vector
vector + α–Fas IgM
sFADD-DN + α–Fas IgM
Yama p32
p17
p12
PARP p116
p85

FIG. 9D

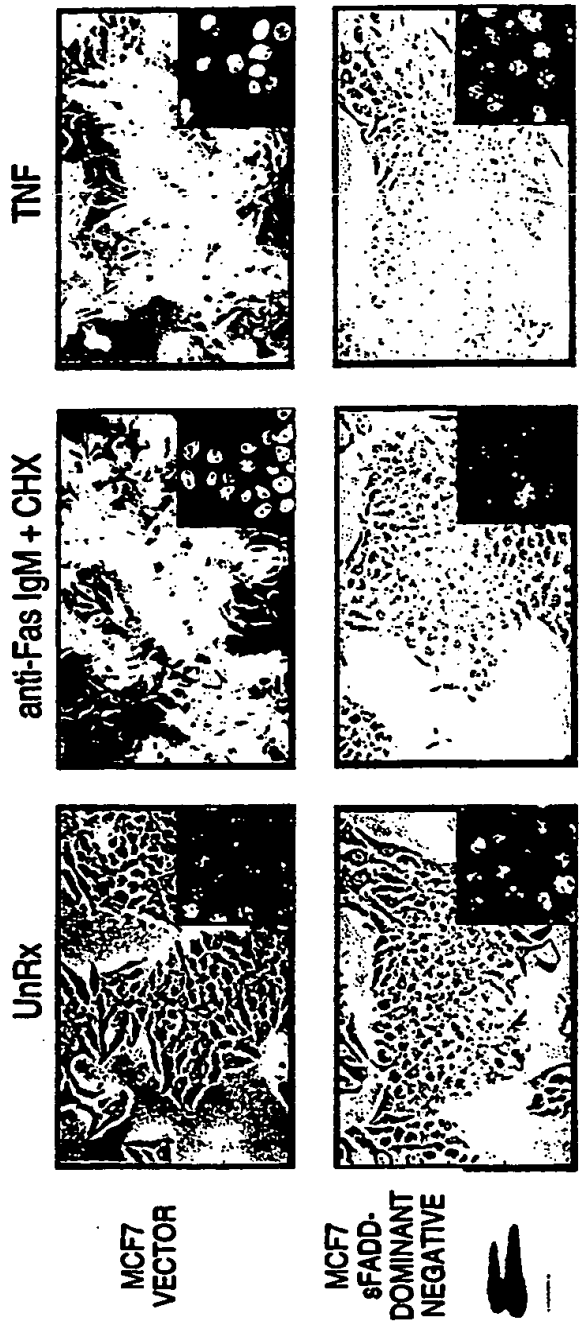
TNF
anti-Fas IgM + CHX
UnRx
MCF7 VECTOR
MCF7 sFADD-DOMINANT NEGATIVE

FIG. 10A

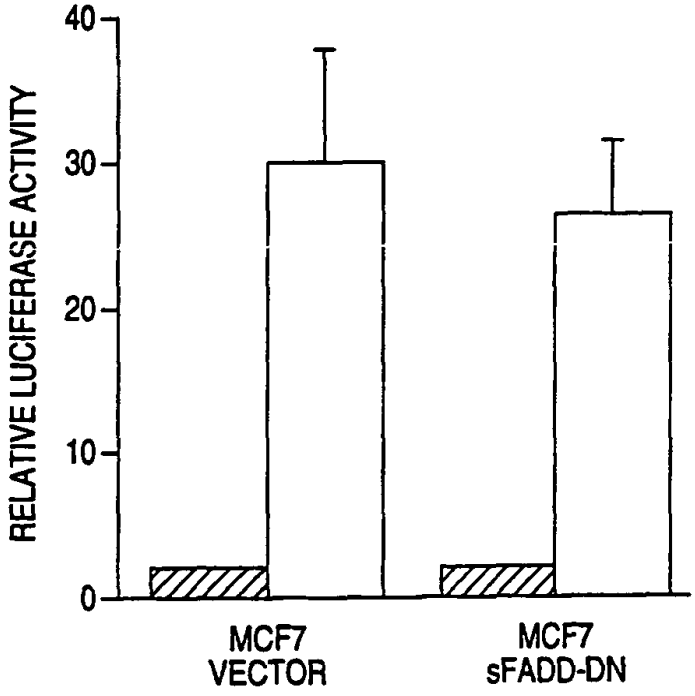
RELATIVE LUCIFERASE ACTIVITY
40
30
20
10
0
MCF7
VECTOR
MCF7
sFADD-DN

FIG. 10B

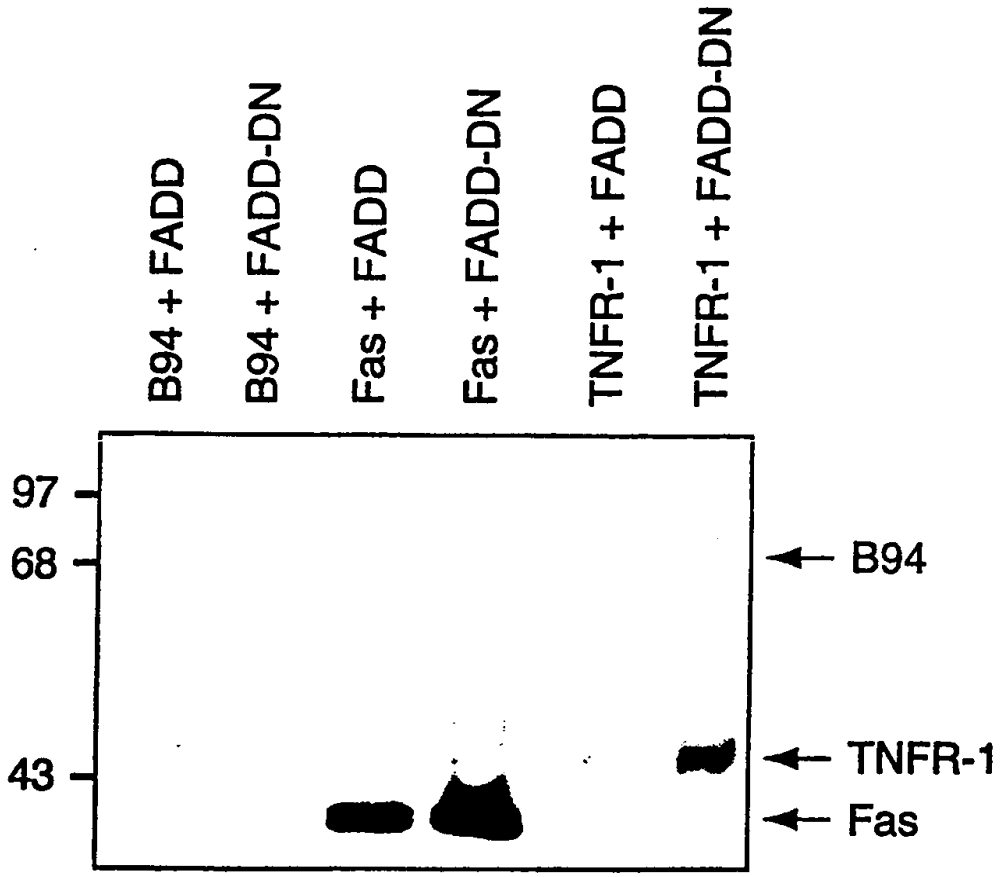
B94 + FADD
B94 + FADD-DN
Fas + FADD
Fas + FADD-DN
TNFR-1 + FADD
TNFR-1 + FADD-DN
97
68
43
B94
TNFR-1
Fas

FIG. 11A

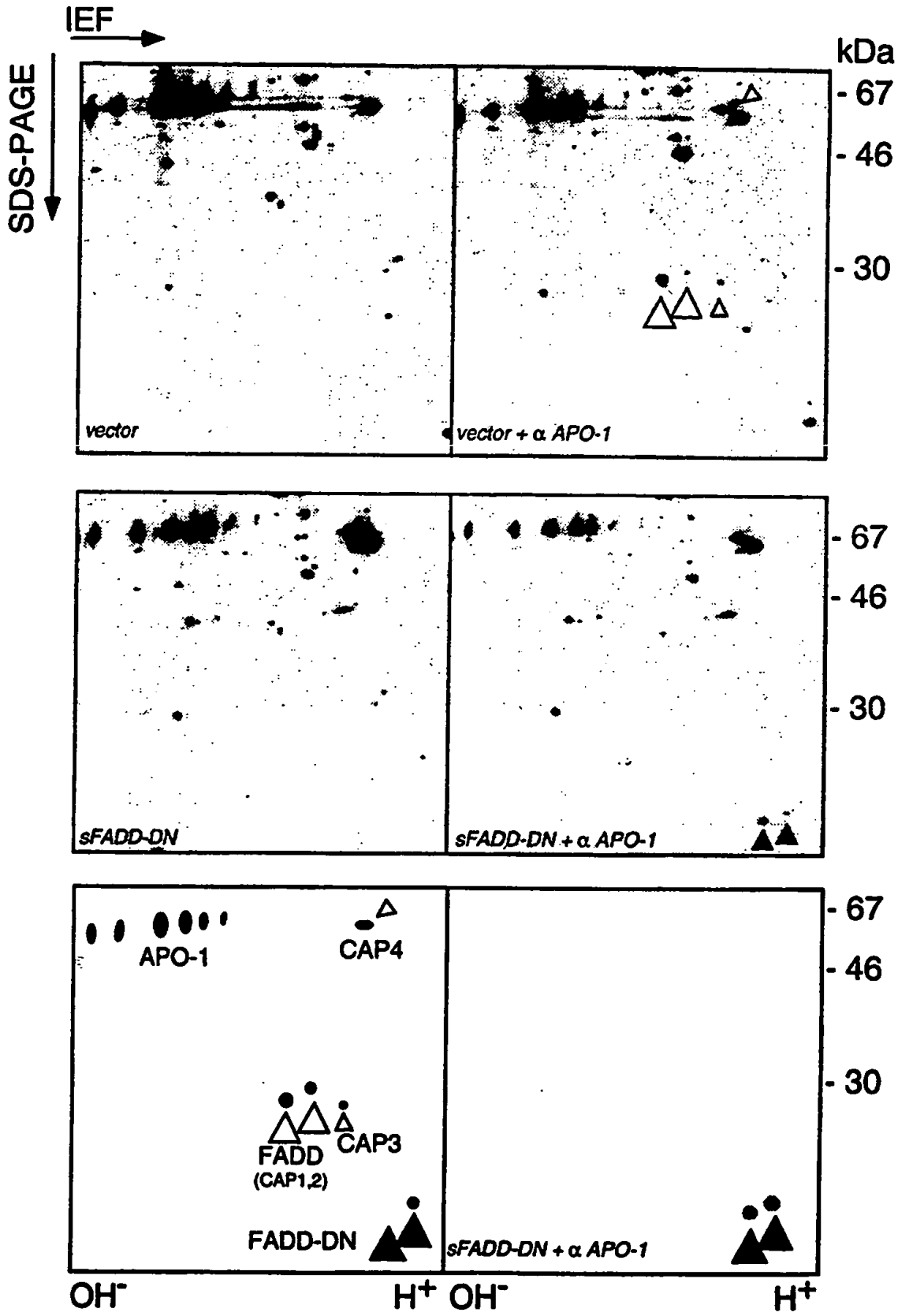
IEF
SDS-PAGE
kDa
- 67
- 46
- 30
vector
vector + α APO-1
sFADD-DN
sFADD-DN + α APO-1
APO-1
CAP4
FADD
(CAP1,2)
CAP3
FADD-DN
sFADD-DN + α APO-1
OH-
H+
OH-
H+

FIG. 11B